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

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Statement of clinical relevance: Early detection of malignant transformation in oral dysplasia leads to simpler treatment of oral cancer. The status of DNA double strand break sensing, signaling and repair pathways can serve as a prognostic indicator in the management of OED.

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Abstract

Introduction: Predicting malignant transformation in oral epithelial dysplasia(OED) is challenging. The higher rate of malignant transformation (MT) reported in non-smokers suggests an endogenous etiology in oncogenesis. We hypothesize that loss of FANCD2 and associated proteins could influence genomic instability and MT in the absence of environmental carcinogens.

Methods: Longitudinal archival samples were obtained from 40 individuals with OED, from diagnosis to the most recent review in 23 stable OED; or until excision of the SCC in 17 unstable OED undergoing MT. Histopathological reassessment, immunohistochemistry for FANCD2 and Western blotting for phosphorylation/monoubiquitylation status of ATR, CHK1, FANCD2 and FANCG were undertaken on each tissue sample.

Results: Decreased expression of FANCD2 was observed in the diagnostic biopsies of OED lesions which later underwent MT. Combining the FANCD2 expression scores with histological grading more accurately predicted MT(p=0.005) than histology alone, and correctly predicted MT in 10/17 initial biopsies. Significantly reduced expression of total FANCD2, pFANCD2, pATR, pCHK-1 and pFANCG were observed in unstable OED.

Discussion: There is preliminary evidence that defects in the DNA damage sensing-signaling-repair cascade are associated with malignant transformation in OED. Loss of expression of FANCD2 protein in association with higher histologic grade dysplasia offered better prediction of MT when compared to clinicopathologic parameters alone.

## Introduction

In the management of oral epithelial dysplasia (OED), histopathologic grading and clinical determinants of malignant transformation to oral squamous cell carcinoma (OSCC) have been the primary influence in the treatment approach adopted 1-3, although various studies indicated putative molecular and other predictors of malignant change 4-6. Most studies aiming at identifying a histopathologic or molecular marker of malignant transformations have not considered correlation with longitudinal clinical outcomes, which diminishes their clinical application 7-9. Furthermore, most studies have not been formally validated in independent series. This approach is limited by the low rate of malignant transformation and a rather prolonged transformation time, leading to a requirement for prolonged studies3. The clinical outcomes of a cohort of patients managed in the Liverpool Multidisciplinary Oral Dysplasia Clinic, identified non-smoking status and the non-homogenous clinical appearance of OED as the strongest independent predictors of malignant transformation (Hazard ratios 5.9 and 2.3 respectively) 1. The estimated malignant transformation rate in this study was 22% over 5 years. The more aggressive behavior of lesions from non-smoking patients (or light smokers) with OED supports an endogenous etiology and although seemingly counter-intuitive, this trend has been seen in other settings of carcinogenesis 10,11.

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 12,13 and occurs earlier in life. The Fanconi Anaemia pathway (FAP) removes inter-strand crosslink (ICL) lesions, 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 14-16. The FAP consists of 22 proteins (FANCC – FANCW) 17: briefly, a FANCM-FAAP24-MHF1-MHF2 complex senses ICLs and localizes to the DNA, acting as a recruitment site for a core complex through FANCM-FANCF interactions. The main role of the core complex is to facilitate ubiquitination of FANCD2-FANCI dimers through FANCL E3 ubiquitin ligase activity, which then activates downstream effector proteins that complete DNA repair and attenuate FAP signaling. Simultaneously, FANCM promotes the ataxia telangiectasia rad–3 (ATR) kinase and checkpoint 1 (CHK1) kinase checkpoint responses which also lead to activation of key components of the FA pathway via phosphorylation at several functional residues, including:- FANCA at serine (S)1449; FANCM at S1045; FANCD2 at S717, S222, S331 and threonine (T)691; FANCG at S387, S383 and S7; and FANCE at S374 and T346 18-21. Left unrepaired, ICL lesions can lead to stalling of replication forks and, eventually, the formation of double-strand breaks effecting genomic instability 22.

We hypothesize that in transforming OED (particularly in non-smokers), aberrations in the DNA damage sensing, signaling or repair pathways influences accumulation of mutations and malignancy. 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 in OED samples, specifically to elucidate if evidence could be obtained from the initial diagnostic biopsy for use as a predictive tool to influence clinical management, and to correlate the findings with clinicopathologic characteristics.

## Methods

Patients**,** Tissue Samples

Forty patients with OED, were identified from a cohort, the clinical characteristics of which have previously been published 1. The patients were included in this study after giving written informed consent and the NHS Research Ethics Committee approved that the study was run in compliance with the Helsinki Declaration (REC ref: EC47.01).

The tissue samples obtained from these patients consisted of incisional biopsies obtained initially to diagnose OED and subsequently at various clinical time points to assess progress of OED to OSCC. They had been routinely fixed and processed for histology to produce formalin-fixed paraffin-embedded (FFPE) blocks. Diagnoses were rendered on 4-5 µm sections from the blocks stained with hematoxylin and eosin (H&E) by two certified, experienced, oral and maxillofacial pathologists; OED had been initially graded as mild, moderate or severe 23,24, but a binary score (group 0: severe dysplasia; group 1: mild or moderate dysplasia) was then endorsed.
Except for the HE sections, the FFPE blocks which were selected had not been used for prior translational investigations and the initial diagnostic tissue size on them had to be sufficient to allow cores to be obtained for protein extraction and Western blotting.

The analysis of the cohort established 23 cases of stable, non-transforming OED (OED-NT) and 17 cases of unstable, malignant transforming OED (OED-T) (1). Archival FFPE blocks of OSCC could not be retrieved in 2 out of the 17 OED-T cases.

### FANCD2 Immunohistochemistry

4 µm FFPE sections from each clinical time point, including the initial diagnostic biopsy, for the 40 patients were stained with an anti-FANCD2 antibody (F117: sc-20022, Santa Cruz, Dallas, USA, diluted 1:100) and the immune complexes were visualized with the use of a Biogenex Supersensitive Polymer HRP detection kit (Launch Diagnostics, Kent, UK, QD430-XAKE) as previously described 11. Expression of FANCD2 was independently assessed by two observers (AT and MH), one being an oral and maxillofacial pathologist. The stained sections were identified by their histopathology report number alone, the observers being thus blinded to the clinical characteristics and transformation outcome. The immunostaining was assessed with regard to cellular localization, extent and intensity; and a descriptive immunohistochemical and histologic, scoring system was developed (see Results and Table 1). Any discrepancy in scoring was reviewed jointly and a mutually agreed score determined. Control tissues from histologically normal areas around the tumour of an OSCC, usually lying at a distance of ≥5mm from it, from a different cohort of anonymized patients (n=3) were similarly stained and assessed.

### Western blotting

The expression of β-actin, ATR, pATR (at S428), CHK1, p-CHK1 (S317), FANCD2 (non-and mono-ubiquitinated isoforms), pFANCD2 (S331), FANCG and pFANCG (S7) were assessed blinded to clinicopathologic details and OED outcome, by western blotting. Areas with the highest grade of dysplasia in the diagnostic, incisional biopsies were marked on the H&E sections and 0.6mm cores were extracted from the corresponding areas of the FFPE blocks (minimum of 2 cores from each block), from all 40 patients. Cores of the same diameter were obtained from normal tissue from esophagus (n=5) for use as a normalizer between gels, OSCC from a different cohort (n=3) and histologically normal areas around the tumour, usually lying at a distance of ≥5mm from it (n=3).

The protein extraction protocol applied was modified from Addis *et al.* 25. Briefly, the FFPE cores were deparaffinized by incubation at room temperature in xylene then rehydrated with a graded series of ethanol. Protein extraction was undertaken using high temperature incubation in Laemmli sample buffer and the samples stored for at −20°C prior to SDS-polyacrylamide gel (PAGE) electrophoresis on 15% gels. The protein was then transferred to a nitrocellulose membrane 26 which was blocked in LI-COR (Li-Cor Biosciences, Cambridge, UK) buffer, to prevent nonspecific binding of the detection antibodies, prior to the addition of specific antibodies at the specified dilutions (Table 2). Anti-mouse or anti-rabbit fluorescently labelled secondary antibodies were utilised at 1:10000 dilution (Alexa Fluor Plus 680 or 800, Invitrogen, Paisley, UK) (Table 2) 20,27-34 followed by visualization of band intensity using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Cambridge, UK). Densitometry values of band intensities were measured and calculated using ImageJ®. These values were normalized for each protein and each sample against both the corresponding β-actin signal and the normal esophageal tissue35 that was loaded onto each gel as a positive control and normalizer (<https://www.proteinatlas.org/ENSG00000144554-FANCD2/tissue>). The values were then ranked and divided into three equal tertiles representing low expression, moderate expression, and high expression (Figure 4). As the specimens from incisional biopsies of OED were much smaller in comparison with those obtained from resections of OSCC, the Western blots were performed on a single occasion due to the limited quantity of available protein.

### Data/Statistical Analysis

Mann Whitney and Fisher exact tests were used to compare the immunohistochemical and histologic scores of the OED-NT and OED-T lesions. The Fisher-Freeman-Halton exact test was used as a measure of significant difference and comparison between the densitometry ratios of OED-NT and OED-T samples from Western blotting. 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 40 patients included in this study are summarized in Table 3. As previously published1 , the malignant transforming (OED-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 (OED-NT) 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 (group 0) compared with 3/23 (13%) non-transformers (p=0.189). These 3 OED-NT patients were progression-free for 18, 52 and 107 months, respectively, following diagnosis and were monitored clinically. The median time to malignant transformation for OED-T in patients with the higher risk binary score (group 0) at diagnosis was 14.8 months when compared with OED-T with a low-risk binary score (group 1), 44.7 months (p = 0.1).

### FANCD2 immunohistochemistry

The histologically normal, oral epithelium adjacent to dysplastic areas did not show any immunostaining (Figure 1A). The dysplastic epithelium *per se* showed variable, moderate to strong, nuclear and cytoplasmic staining (Figure 1A, B). Staining was absent in areas of microinvasion (Figure 1C, D) and did not seem related to the intensity or pattern of inflammatory reaction in the underlying stroma (Figures 1 and 2). In OSCC derived from OED-T, both intensity and distribution of the staining were reduced compared with the initial diagnostic biopsy from the same patient (Table 4).

The localization, intensity and distribution of the immunostaining in OED-NT, OED-T and paired OSCC are detailed in Table 4. A decreased intensity of both nuclear and cytoplasmic FANCD2 staining was observed in the initial, diagnostic biopsies of OED-T when compared with OED-NT (Figure 2), but the distribution of staining was not statistically different (Table 4). On this basis, the quantitative rather than qualitative quality of the staining was deemed worthy of further analysis.

A composite FANCD2-Histology OED score was devised whereby three characteristics were assigned a binary code of 0 or 1 and the scores were summed to give the final value. The variables scored were FANCD2 nuclear intensity of immunostaining, FANCD2 cytoplasmic intensity of immunostaining and histologic assessment of the OED epithelium. Specifically, absent/weak nuclear or cytoplasmic immunostaining and histologically severe dysplasia/carcinoma-in-situ/OSCC were each assigned 0; moderate/strong nuclear or cytoplasmic stain and histologically mild/moderate dysplasia were each assigned 1. As an example, a case of severe dysplasia (0) with absent/weak nuclear staining (0) and moderate/strong cytoplasmic stain (1) would yield a summation score of 0 + 0 + 1 = 1.

Further statistical analysis showed that the FANCD2-Histology OED summation score was confidently related to malignant transformation (p=0.005) (Table 5), a summation value cut-off of ≤1 being significantly associated with a higher risk of transformation (p=0.001). Eighty-three percent (10/12) of patients with a low (≤1) score across the whole cohort went on to develop OSCC compared with 25% (7/28) of patients with a high (≥2) score. The diagnostic biopsies for the 7 OED-T lesions with a ≥2 score were taken a median of 5.8 years (range 0.3-7.0 years) prior to the date of malignant transformation, whereas those from the 10 OED-T lesions with a ≤1 score were taken a median of 2.4 years prior to malignant transformation (range 0.2-5.3 years). Of the 2 OED-NT lesions with a score ≤1 (Table 5), one corresponded to a severe dysplasia which was promptly excised, the patient then being progression free for 52 months; and the other to a mild dysplasia which has been progression-free for 19 months. In all 15 of the available OSCCs which had arisen from OED-T, the reduced FANCD2 immunostaining mentioned above was reflected in a correspondingly low score (Table 5).

The FANCD2-Histology OED score was 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; though, predictably, was associated with grade of dysplasia (p<0.0005). However, the FANCD2-Histology OED score was a better predictor of transformation than histology alone, with 5/12 dysplasia of low to moderate grade being correctly predicted as transformers by the FANCD2-Histology OED score.

### FANCD2 and associated protein expression

No significant differences in the normalized expression of β-actin, ATR, CHK1 and FANCG were observed between OED-T and OED-NT lesions (Table 6; Figures 3 and 4). Significantly reduced expression of FANCD2 (non-ubiquitinated (FANCD2-S) and mono-ubiquitinated (FANCD2-L) forms), pFANCD2, pATR, pCHK1 and pFANCG were observed in OED-T prior to malignant transformation, with the FANCD2, pFANCD2 and pFANCG observations mirrored in the control OSCC tumour tissue. These parameters did not correlate with any clinicopathologic 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 OED-NT and OED-T, respectively (p < 0.001).

## Discussion

The propensity for patients with the cancer prone syndrome, Fanconi Anaemia, to increased incidence of HNSCC 12,13 and the evidence that individuals with reduced, systemic, double strand break repair capacity are more prone to develop head and neck cancer16 both suggest a role for this pathway in HNSCC. It has further been suggested that activation of the DNA damage response might be protective in the early stages of oral carcinogenesis, but that progressive deregulation over time could eventually result in the failure to suppress malignant transformation36. Our hypothesis that malignant change in OED involves alteration of the FA pathway is supported by both the immunohistochemical and western blotting data presented here.

We observed an increase in FANCD2 protein expression in OED followed by its loss at the point of malignant progression. Such a loss should thus be considered within the context of the presence of OED (or OSCC) and supports including the grade of OED into the FANCD2-Histology OED score. In the small cohort of histologically normal oral epithelium (obtained from the margins of resected OSCC specimens) used in this study, expression of FANCD2 was not observed. This suggests that, in the absence of OED or OSCC, the lack of FANCD2 expression would indicate only that the Fanconi Anaemia Pathway has not been activated rather than it has been de-activated.

In relation to prediction of malignant transformation, Rudland et al 11 suggested that cytoplasmic as well as nuclear FANCD2 immunostaining may be prognostic for breast cancer. Their scoring system also included extent and intensity of immunostaining11. We felt that extent of staining would not be appropriate for our OED samples which were small in comparison to resections. In the present study, the intensity of IHC staining for FANCD2 coupled with a histologic binary dysplasia score correctly predicted malignant transformation in 10/17 (59%) diagnostic biopsy samples obtained prior to transformation, with a ‘false positive’ rate of 2/23 (9%) for OED-NT biopsies. This performed better than dysplasia grading alone, either as a binary score or as the current WHO classification24. The significantly different rates of malignant transformation observed between patients with low intensity FANCD2 (10/12: 83%) expression and higher intensity FANCD2 (7/28: 25%) expression further support its potential for assessing the risk of carcinogenesis. In turn, the value in the novel score may be in better identifying lesions which will not transform, itself a potentially valuable clinical stratification for affected patients.

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 discussion in the literature suggests that such binary classifications are simplistic 37,38. Attempts at binary or other classifications of OED largely rely on histopathologic interpretation, which are influenced by intra and inter-observer reliability problems 39,40. It is acknowledged, therefore, that the proposed FANCD2-Histology OED classification will have skewed the moderately dysplastic lesions in this study towards a lower risk score, although still performing better than histopathology alone. The median follow-up period was less in the OED-NT vs the OED-T group (3.4 vs 6.9 years, respectively), and this may account for the 2 OED-NT lesions with low (≤1) FANCD2-Histology OED scores and the 7 OED-T lesions with high (≥2) FANCD2-Histology 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 our technique for predicting malignant transformation prior to the event. For example, of the 6 OED-T patients in whom we had non-neoplastic tissue from an intermediate time point, we observed that the FANCD2-Histology OED scores decreased in 2 cases (from ≥2 to ≤1) and remained static in 4 patients (3/4 of whom had already scored ≤1 at the initial biopsy). All 5 of the available OSCC from these patients scored ≤1.

FANCD2 monoubiquitylation, which is thought to be promoted by ATR-CHK1 mediated FANCD2 phosphorylation 19,31, is a critical step in FA pathway activation 30,41 and evidence suggests that a reduction in FANCD2 monoubiquitylation has a greater influence on genomic instability than down regulation of FANCD2 expression 42. In our study, it was observed via Western blotting that transforming samples show lower levels of FANCD2 monoubiquitylation (FANCD2-L expression) compared to non-transformers, and interestingly, they consistently showed lower levels of total FANCD2 expression. These findings agree with our immunohistochemical data. Samples from OED-T lesions also showed significant reduction in the phosphorylation of ATR, CHK1, FANCD2 and FANCG in comparison to OED-NT samples, but levels of the unphosphorylated proteins were largely unchanged indicating a lack of ATR-CHK1 activation. It may be argued that the observed reduction in activating post-translational modifications, such as phosphorylation and monoubiquitination, is attributable to a lack of environmental stimuli in the transforming group which has a preponderance of non-smokers, but our data does not support this as no significant difference in expression of post-translational modifications was observed between smokers and non-smokers. Thus, the ability of CHK1 to phosphorylate several functionally important sites for optimal function and activation of the FA pathway appears to be compromised in OED-T patients, which, could lead to impairment of the functionality of the FA core complex and a reduction in subsequent homologous recombination repair activity 20,27,30,43. In contrast, the relatively higher levels of ATR, CHK1, FANCD2 s331 and FANCG S7 phosphorylation in OED-NT, suggest successful activation and effective DNA repair, thus reducing the burden of DNA damage in the cells and in turn the risk of malignant transformation. This statistically significant reduced expression of post-translationally modified proteins in the DNA repair pathway in OED-T when compared to OED-NT seems a more accurate predictor of malignant transformation than clinical parameters such as smoking history and site. However, it should be noted that site, smoking etiology and appearance were such strong predictors of transformation in our modest cohort that it was impossible to adequately match the two groups.

The present study is best categorized as proof-of-concept as the design does not support sufficient statistical power for a validated prognostic biomarker study. The value of the FANCD2-Histology OED score, with or without additional post-translational modification markers, may principally be in identifying lesions which will not transform. In this group of patients, there is potential for their OED lesions to be managed conservatively, be subjected to less frequent follow-up and discharged to primary care for surveillance after a period of stable clinical review in secondary care. However, development into a potential biomarker for malignant transformation in OED requires validation of IHC staining categorization beyond the scope of this manuscript 44. In common with other similar studies, another difficulty is whether a small diagnostic biopsy is as representative of the lesion’s biology as a complete resection (the latter procedure denying unfettered longitudinal analysis for progression). Clinical experience, where biopsy of OED is carried out by senior surgical members of the team who sample the area of most clinical concern, suggests that concordance in histology between the initial diagnostic biopsy and final definitive resections is usually good 45.

Validation of this putative FANCD2-Histology OED score requires a larger sample size to better control for potentially confounding variables by matching for smoking, clinical appearance, age, and management (surveillance vs excision). Indeed, 6 of the 23 OED-NT lesions in this study were totally excised, therefore some of the differences observed between OED-NT and OED-T groups might relate to the method of treatment rather than the inherent cancer risk of the sample.

It has been suggested that phosphorylated proteins are more labile, and that epitope degradation can occur within 30 minutes of ischemia in formaldehyde 46. However, we have demonstrated differential down-regulation of 4 different phosphorylation sites in OED-T compared with OED-NT. This finding is significant given the lack of availability of fresh tissue collections from cohorts of dysplasia patients and suggests that phosphorylation biomarkers of transformation may be developed for FFPE tissue. This could potentially be used to influence treatment decisions in clinical practice or when used within the context of a clinical trial in the management of OED to stratify treatment/intervention arms.

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 OED47-49 and there is evidence to suggest that LOH is secondary to homologous recombination deficiency/DNA damage repair at 15 cancer sites, including HNSCC 50. This is a possible mechanistic link to our hypothesis and the putative FANCD2-Histology OED score might conceivably be enhanced by incorporating LOH 51.

Conclusion

Our data have demonstrated proof-of-principle for a novel FANCD2-Histology OED score in evaluation of the status of the Fanconi Anaemia Pathway in OED. Further we have shown its potential for clinical translation as a predictive biomarker of malignant transformation, particularly in identifying patients with lower risk OED. These findings require validation in a larger cohort prior to clinical application. We have identified novel mechanistic insights into specific aberrations in DNA repair, potentially explaining the paradox as to why so many OED patients *without* exposure to environmental carcinogens demonstrate such high risk for malignant transformation.

Abbreviations:

NT – Non-transforming / stable

HNSCC ­– Head and neck squamous cell carcinoma

OED – Oral epithelial dysplasia

OSCC – Oral squamous cell carcinoma

T – Malignant transforming / unstable

Declarations:

Ethical approval and consent to participate for publication: Ethical approval for this study was given by Sefton REC (now North West – Liverpool Central; reference number: EC 47.01). Written consent was obtained for retrospective and prospective analysis of tissue samples and clinical records.

Availability of data and material: Anonymized data presented in this study will be made available on written request to the corresponding author for future systematic review and/or metanalysis

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Table 1. FANCD2 descriptive, binary immunohistochemistry scoring system.

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

Table 2. Antibodies used in Western blot.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Antibody against** | **Raised in** | **Company** | **Catalogue number** | **Dilution** | **Citations** |
| β-actin (C4) | Mouse | Santa Cruz | sc-47778 | 1:5,000 | 28 |
| ATR | Rabbit | Atlas | HPA028264 | 1:1,000 | 29 |
| pATR | Mouse | Gift of Dr G. M. Kupfer | 1:1,000 | 27,30 |
| CHK1 | Mouse | Santa Cruz | sc-56291 | 1:1,000 | 31 |
| pCHK1 (S317) | Rabbit | Calbiochem | DR1025 | 1:1,000 | 31 |
| FANCD2\* | Mouse | Santa Cruz | sc-28194 | 1:500 | 20 |
| pFANCD2\* (S331\*) | Mouse | Gift of Dr G. M. Kupfer  | 1:1,000 | 27,30 |
| FANCG\* | Rabbit | Santa Cruz | sc-28219 | 1:1,000 | 20 |
| pFANCG\* (S7\*) | Mouse | Gift of Dr G. M. Kupfer  | 1:1,000 | 30,32 |

\*selected as markers of activation of the Fanconi Anaemia pathway availability and reliability from previously published data20,34

Table 3. Demographic and clinic-pathologic features of patients (n = 40).

|  |  |  |  |
| --- | --- | --- | --- |
|  | Non-transforming (NT) (n =23) | Transforming (T)(n=17) | p value |
| Age | Median (range) | 55.9(27.9 – 78.4) | 57.5 (37.5-85.2) | 0.56 |
| Gender n (%) | MaleFemale | 13 (57)10 (43) | 6 (35)11 (65) | 0.22 |
| Site n (%) | Lateral tongueFloor mouthBuccalAlveolar mucosa | 4 (17)11 (49)4 (17)4 (17) | 10 (58)3 (18)4 (24)- | 0.01 |
| Appearance n (%) | Homogenous whiteRed-whiteRed | 20 (87)2 (9)1 (4) | 7 (41)10 (59)- | 0.001 |
| Lesion size n (%) | < 200mm2> 200mm2 | 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 (%) | TeetotalCurrent drinker | 6 (26)17 (74) | 5 (29)12 (71) | 1 |
| Grade of dysplasia\*n (%) | Mild/moderateSevere | 20 (87)3 (13) | 12 (71)5 (29) | 0.19 |
| Definitive treatment  | SurveillanceExcision | 17 (74)6 (26) | 017 (100) | 0.0001 |

\* at initial diagnostic biopsy

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

|  |  |  |
| --- | --- | --- |
|  |  | Biopsy |
| Site of staining | Pattern of staining | NT - first diagnosticn (%) | T – first diagnosticn (%) | T – OSCC diagnosticn (%) |
| 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) |

\* 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 5. FANCD2-Histology OED Risk Scores in stable OED (NT), OED which underwent malignant transformation (T) and oral squamous cell carcinoma which had arisen from OED.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| FANC D2–OED Risk Score  | OED-NTn = 23 | OED-Tn = 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 |  | OSCCn = 15 |  |
| 1 |  |  | 7 | 15 |
| 0 |  |  | 8 |

\* Statistical significance when a summation value cutoff of ≤1 was applied

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

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Normalized densitometry score ratio\* | Low expressionn (%) | Moderate expressionn (%) | High expressionn (%) | p value(Fisher-Freeman-Halton exacttest) |
| ATR | TNT | 97 | 38 | 58 | 0.24 |
| pATR  | TNT | 141 | 39 | 013 | < 0.0005 |
| Chk1 | TNT | 510 | 75 | 58 | > 0.29 |
| pChk1  | TNT | 133 | 38 | 112 | < 0.0005 |
| FANCD2 (S & L#) | TNT | 1010 | 28 | 55 | < 0.025 |
| pFANCD2-S331  | TNT | 161 | 112 | 010 | < 0.0005 |
| FANCG | TNT | 410 | 64 | 79 | 0.61 |
| pFANCG-S7 | TNT | 161 | 110 | 012 | < 0.0005 |

* See materials and methods

# Sum of ‘small’ and ‘large’ FANCD2 isoforms - L corresponds to the mono-ubiquitylated (activated) isoform

p: phosphorylated proteins; S331 and S7: location of phosphorylation

Figure 1**.** Immunohistochemistry for FANCD2. The photomicrographs illustrate the transition between normal (non-dysplastic; n) and dysplastic (d) epithelium (A & B); and between dysplastic (d) epithelium and early squamous cell carcinoma (t) (C & D). Differences in expression of FANCD2 between normal, dysplastic and invasive epithelia are seen. While variably strong, nuclear and cytoplasmic immunostaining is centered on the suprabasal layers of dysplastic epithelia, it is absent from normal epithelium (n in A) and lost from the tumour (t in C). Basal keratinocytes of the dysplastic epithelia are increased in numbers (A) or show perturbed stratification and nuclear hyperchromatism (B). Inflammatory reaction (i) of variable density is seen underneath dysplastic epithelia and tumour (A & C). 1B & 1D are from adjacent sections stained with H & E to allow comparison. Objective magnification × 4.

Figure 2**.** Immunohistochemistry for FANCD2 in OED. The photomicrographs illustrate differences in expression of FANCD2 between OED-NT (A & C) and OED-T (E & G). Although variably strong immunostaining decorates non-keratinizing layers in OED-NT (A & C), it is absent from basal and parabasal, epithelial components (asterisks) in OED-T (E & G). Higher magnification allows appreciation of the differences and dysplastic features (C, D & G, H), including swollen, drop-shaped rete, perturbed stratification, enlarged nuclei (arrow), increased nuclear : cytoplasmic ratio (C, D), a markedly irregular lower epithelial border and increased numbers of cells having a basaloid phenotype (G, H). Note the variable inflammatory reaction. B, D, F & H are from adjacent sections stained with H & E to allow comparison. Objective magnification × 4 (A, B, E, F); × 10 (C, D, G, H).

Figure 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 esophagus; CT: control OSCC from a different cohort; Molecular weights (kDa): β-actin(42), ATR(250), pATR(250), Chk1(54), pChk1(56), FANCD2 (164) and FANCG (69).

Figure 4. Normalized western blotting data for all samples and all antibodies.

All: all samples (n=48); Normal: normal adjacent tissue (n=3); NT: OED-NT samples (n=23); T: OED-T (n=17); Cancer: OSCC (n=3). Blue dashed lines represent the equal tertiles into which each data set was divided