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ANATOMICAL PATHOLOGY

Tumour mismatch repair protein loss is associated with advanced stage in oral cavity squamous cell carcinoma

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Summary

An unexplained increase in the incidence of oral cavity squamous cell carcinoma (oSCC) has been observed despite decreasing smoking rates, particularly in younger patients. Links to defects in the DNA mismatch repair (MMR) system are well established in early onset colorectal, urothelial and gynaecological malignancies. MMR deficient patients treated with immune checkpoint inhibitors have demonstrated improved response rates. Studies exploring MMR status in head and neck squamous cell carcinoma (HNSCC) demonstrate conflicting results. This study explores the incidence of MMR protein loss and its association with clinicopathological features and outcome in oSCC.

Immunohistochemical staining using tissue microarrays to assess the expression of MMR proteins (hMLH1, hMSH2, hMSH6, and hPMS2) was performed on 285 consecutive oSCC cases between 2000 and 2016. Data on smoking, alcohol and metachronous malignancies were retrospectively collected. Proportional hazards regression models were used to compare survival in MMR intact and deficient patients.

MMR deficiency was seen in 21 patients (7.4%). MMR deficient tumours were associated with bone invasion (52% vs 32%, p=0.05), higher pT stage (pT4 in 57% vs 35%, p<0.001) and a higher number of metachronous malignancies (p=0.05). MMR deficiency was not associated with younger age at presentation or absence of smoking or alcohol. There was no significant association between MMR status and survival (overall survival hazard ratio 1.36; p=0.32).

The incidence of MMR loss in oSCC is low and is not associated with young age at presentation. MMR deficiency in oSCC is associated with an increase in the number of metachronous malignancies and more advanced primary tumours.

Key words: Oral head and neck squamous cell carcinoma; oral cancer; head and neck cancer; oral cavity; MLH1; MSH2; MSH6; PMS2; mismatch repair; immune checkpoint inhibitors.

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INTRODUCTION

Oral squamous cell carcinoma (oSCC) is the sixth most common cancer worldwide.[1] There appears to be an increasing incidence in oSCC distinct from human papillomavirus (HPV) related oropharyngeal squamous cell carcinoma.[2–5] In developed nations, many of these tumours are occurring in the context of minimal exposure to traditional risk factors such as alcohol and tobacco.[6] The cause for this is unknown but there may be an underlying genetic cause or an unknown environmental agent contributing to the rising incidence of these tumours.

The DNA mismatch repair (MMR) system fosters genomic stability by utilising specialised proteins for post-replicative repair of base mispairs and insertion/deletion loops. The MMR system includes two complexes; MutSα, a heterodimer composed of two proteins, *hMSH2* and *hMSH6* and MutLα heterodimer complex composed of *hMLH1* and *hPMS2*. Mismatch recognition is carried out by MutSα. The MutLα heterodimer complex creates a nick in the DNA, allowing erroneous strands to be degraded and repaired. Loss of MMR function in cells leads to accumulation of replicative errors and the expression of a 'mutator' phenotype, distinguished by increased microsatellite instability (MSI) and higher rates of spontaneous mutations.[7] Germline mutations in MMR have been shown to be associated with young onset malignancies and increased incidence of metachronous malignancies.[8,9] MMR protein loss, both germline and somatic, and its effects have been extensively studied in colon cancer and carry treatment implications, particularly in the form of resistance to platinum based therapy, a standard of care treatment protocol also used in head and neck malignancies.[10,11] Also, recently, MMR deficient patients have been observed to show improved and sustained response on immune checkpoint inhibitors.[12,13]

Given the rising incidence of non-HPV oSCC in non-smokers, there is a need to investigate the mechanisms of carcinogenesis in oSCC. The role of MMR in oSCC is not well established.[14] The literature has reported conflicting rates of protein expression and prognostic implications of this repair pathway in oSCC.[15–30] Limitations exist due to varying methods to assess MMR dysregulation within small cohorts.[27–29]

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The primary aim of our study is to evaluate the incidence of MMR protein deficiency using immunohistochemistry in non-HPV related oSCC. Secondarily, the study aimed to evaluate the prognostic significance of MMR protein deficiency and the incidence of metachronous malignancies in MMR deficient patients.

MATERIALS AND METHODS

Study population

Consecutive patients with oSCC who underwent surgical treatment with curative intent between 2000 and 2016 were identified from the Sydney Head and Neck Cancer Institute (SHNCI) database at Chris O'Brien Lifehouse, Sydney, Australia, following institutional ethics approval. The archival slides and blocks were retrieved from the Department of Tissue Pathology and Diagnostic Oncology at Royal Prince Alfred Hospital. Exclusion criteria included inadequate/unavailable specimens or clinicopathological data, patients with oropharyngeal carcinoma and HPV related carcinoma. In total, 285 patients with non-HPV related oSCC with adequate histological material were available for the study.

Clinicopathological data

The SHNCI database prospectively collects patient clinicopathological parameters including demographics, staging, treatment, histopathological data and follow up details. Further data on metachronous malignancy, smoking and alcohol history were retrospectively collected from patient records. Heavy alcohol use was defined as >210 g/week in males and >110 g/week in females. Heavy smoking was defined as greater than a 10 pack-year history.

Histopathological analysis

A complete histopathological review was performed blinded to patient clinical outcomes. The tumour size, depth of invasion, lymphovascular and perineural invasion, bone involvement and margins of resection were recorded. Tumours were staged pathologically according to the 7th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual.

DNA *in situ* hybridisation studies for high risk HPV were performed[31] and lack of high-risk HPV integration was confirmed.

MMR immunohistochemistry

Immunohistochemistry (IHC) was conducted to assess the expression of MMR proteins (hMLH1, hMSH2, hMSH6, hPMS2). The IHC staining was first performed on whole sections from 24 cases of oSCC to understand the degree of heterogeneity if any in staining, and on the remaining cases using tissue microarrays (TMA). Highly cellular areas of squamous cell carcinoma with a neoplastic cell content of 30–90% without necrosis, keratin, inflammatory infiltrate or haemorrhage were identified for TMA. Two cores, 1 mm in diameter, were obtained from the invasive front of the tumour for each sample to account for the heterogeneity if any in the immunohistochemical staining patterns.[32]

Whole FFPE block tissue sections and tissue microarray sections were cut at 3 µm onto Superfrost+ glass slides and stored at 4°C until IHC was performed (<2 weeks).

IHC staining for MSH2, MSH6, PMS2 and MLH1 was performed using Leica Bond III automated staining platform (Leica Biosystems, Australia), with a high pH target retrieval buffer (pH 9) as per the manufacturer's instructions. The primary antibodies against the MMR proteins (see Table 1 for clones, manufacturers and dilutions) were visualised using Bond Polymer Refine Detection, (DS9800; Leica) as per the manufacturer's instructions.

MMR expression in tumour cells

MMR expression proficient and expression deficient colorectal tumours were used as positive and negative external controls. Proficient MMR protein expression was defined as diffuse strong positive staining within at least 5% of tumour nuclei, using adjacent non-tumoural mucosa and lymphocytes as positive internal controls (Fig. 1).[33] Deficient MMR protein expression was defined as loss of nuclear staining within the tumour as compared to internal and external controls (Fig. 2).[34] Staining evaluation was undertaken by two pathologists (SA and RG) and discrepant cases were reviewed for consensus.

Statistical analysis

The key endpoints were overall survival (OS), disease-free survival (DFS) and disease specific survival (DSS). All survival times were measured from date of surgery. DFS was defined as the time interval between date of surgery and date of disease progression or date of death. The univariate association of MMR status with DFS and OS were investigated using proportional hazards regression models. To investigate the association with DSS, competing risk models were used to take into account death from other causes or death prior to recurrence. The Kaplan–Meier method was used to construct survival curves. Cross-tabulation and a corresponding Fisher's exact test were used to see if there was an association between a history of cancer and immunohistochemical MMR status.

Statistical analysis was performed using Stata version 14.0 (StataCorp, USA) and a p value <0.05 was considered statistically significant.

RESULTS

Cohort characteristics

The study population includes 285 patients with oSCC. The majority of patients were male (n=166, 58.2%). The mean age of the cohort was 63.6 years (SD ±13.6) and there were 49 patients less than 50 years of age. The tongue was the predominant site, with 39.6% of tumours arising from the oral tongue (Table 2). The median follow-up was 6.5 years [95% confidence interval (CI) 5.14–7.29 years]. In addition to surgery, 114 patients received adjuvant radiotherapy, of which 35 received concurrent chemotherapy. During the study period disease recurrence occurred in 72 (25.3%) patients, of which 58 (20.4%) were locoregional, 11 (3.9%) were distant, and 3 (1.1%) were concurrent locoregional and distant. A total of 126 (44.2%) patients died, of which 55 (19.3%) deaths were attributed to head and neck cancer. The demographic and clinicopathological parameters are presented in Table 2.

Immunohistochemical MMR protein expression

Loss of MMR protein expression was observed in 21 cases (Table 3). hMLH1 loss was noted in 12 patients, of which seven demonstrated both hPMS2 and hMLH1 expression loss. hMSH6 expression was lost in in 11 patients and hMSH2 expression was lost in six patients. Of these, three patients demonstrated both hMSH6 and hMSH2 expression loss. There were three patients who demonstrated deficient expression patterns for all four MMR proteins.

MMR and clinicopathological features

The mean age of the MMR deficient group was 65.4 years versus 63.0 years of the entire cohort (p=0.45). A gender predilection was not observed. Loss of MMR expression was associated with bone invasion (52.4% vs 31.8%, p=0.05) and pT4 category (57.1% vs 34.8%, p<0.001) as compared to MMR proficient patients. However, other primary tumour characteristics were similar in both groups, with no significant difference in tumour differentiation, depth of invasion, lymphovascular or perineural involvement, N-category or treatment patterns.

MMR and risk factors

In the study cohort, there were a total of 174 smokers and 63 patients with heavy alcohol consumption. An increased incidence of MMR deficiency in oSCC was not observed

Table 1 Mismatch repair staining method

Antibody	Manufacturer	Clone	Dilution	Retrieval	Detection kit	Instrument
MLH1	Novacastra (Leica)	ES05	1/100	Retrieval Solution 2 (pH 9) for 20 min	Bond Polymer Refine Detection	Bond III, Leica
MSH2	Dako	FE11	1/25	Retrieval Solution 2 (pH 9) for 30 min	Bond Polymer Refine Detection	Bond III, Leica
MSH6	Dako	EP49	1/50	Retrieval Solution 2 (pH 9) for 30 min	Bond Polymer Refine Detection	Bond III, Leica
PMS2	Dako	EP51	1/20	Retrieval Solution 2 (pH 9) for 30 min	Bond Polymer Refine Detection	Bond III, Leica

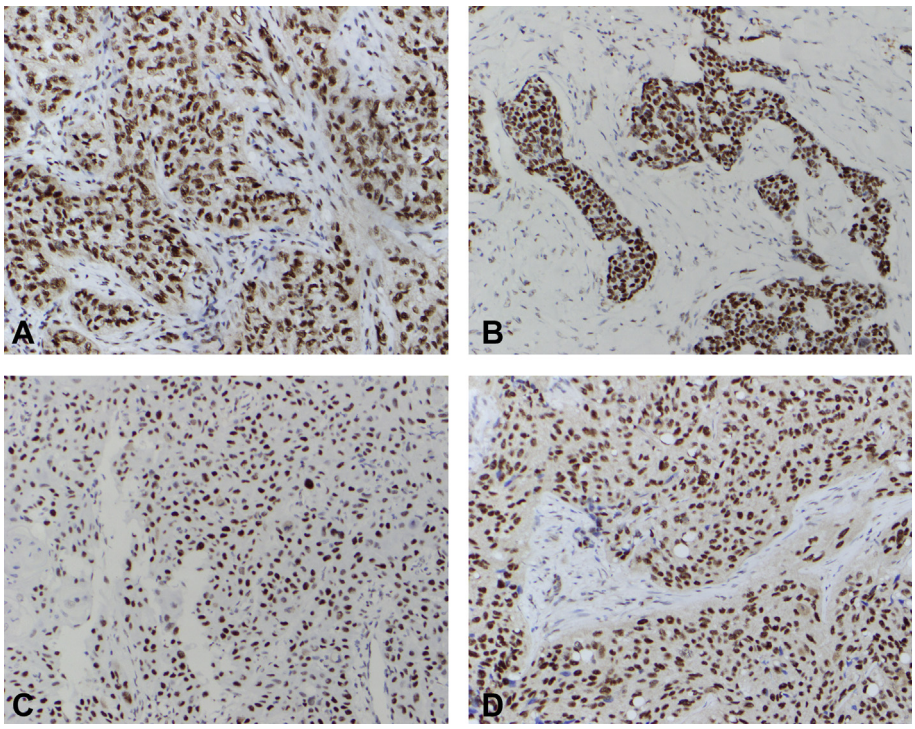

Fig. 1 Proficient immunohistochemistry for MMR. (A) MLH1, (B) MSH2, (C) MSH6, (D) PMS2.

amongst non-smokers or those without alcohol consumption in this cohort.

MMR and metachronous tumours

Information regarding metachronous tumours was available in 135 patients, of which 41 (30.4%) patients developed a metachronous tumour. The mean age of patients who developed metachronous tumour was slightly higher than those who did not (67.8 vs 63.5 years). The average time from oSCC treatment to development of second cancer was 6.8 years (range 0–27 years). Tumours developed prior to oSCC diagnosis pre-dated the oSCC on average by 4 years (range 1–8). Of the 12 MMR deficient patients where reliable information was available, five (41.6%) developed metachronous tumours. Of the 122 MMR normal patients with reliable information, 36 (29.5%) developed a metachronous tumour. However, this difference was not statistically significant (p=0.51 Fisher's exact test).

The most common metachronous tumour sites were breast (n=7), lung (n=7) and prostate (n=6). Gastrointestinal tract neoplasia was present in five patients, colorectal cancer (n=4) and upper gastrointestinal cancer (n=1). In patients with metachronous malignancies, those with MMR deficiency had a higher number of metachronous malignancies, with two or more malignancies demonstrated in three (n=12, 25%) MMR deficient patients compared to five (n=131, 4%) MMR normal patients (p=0.03).

MMR and survival

There was no association between MMR expression loss and OS [hazard ratio (HR) 1.36; 95% CI 0.75–2.46; p=0.32], DFS (HR 1.31; 95% CI 0.76–2.28; p=0.33) and DSS (HR 0.43; 95% CI 0.11–1.70; p=0.23) (Tables 4 and 5).

DISCUSSION

The MMR system is responsible for proof-reading of the DNA during replications. Defects in the MMR system thus leads to accumulation of errors within the DNA, particularly in the repetitive sequences of DNA that are prone to errors when replicated, known as microsatellites. Germline mutations in the MMR genes, as identified in Lynch syndrome, lead to microsatellite instability and an inherited tumour predisposition phenotype in the patient.[12] It is now well established that patients who are non-smokers, non-drinkers and non-HPV carriers are developing oSCC, in particular at the tongue sub-site, at an increasing rate worldwide.[35,36] Identification of a genetic predisposition in this patient cohort would shed light on this alarming trend.

This study including 285 patients is the largest study to date assessing the expression of key MMR proteins in oSCC.

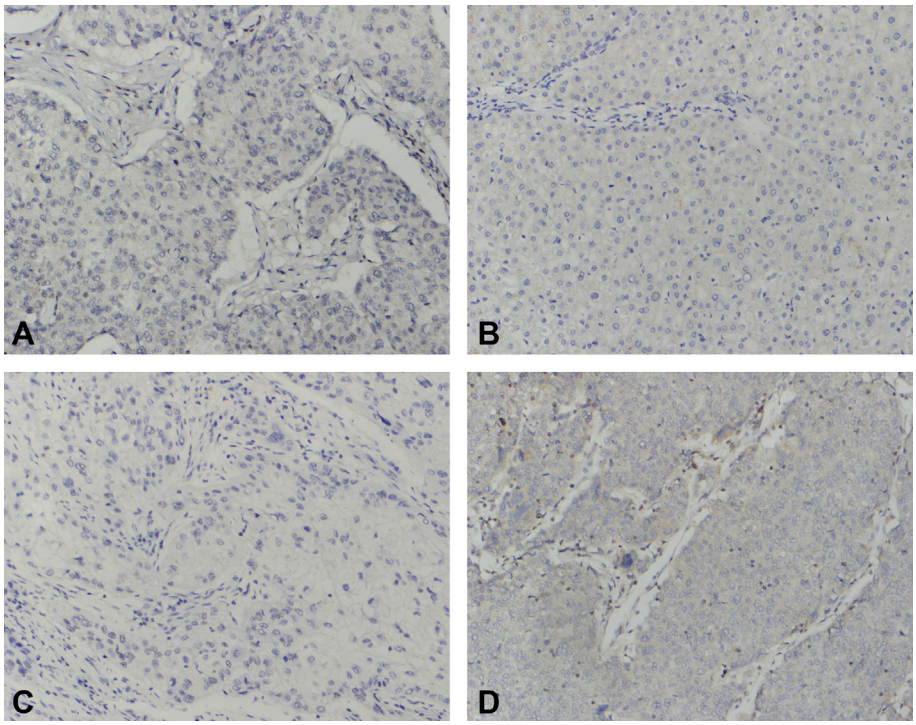

Fig. 2 Deficient immunohistochemistry for MMR. (A) MLH1, (B) MSH2, (C) MSH6, (D) PMS2.

Twenty-one (7.4%) patients demonstrated a loss of expression of at least one MMR protein and nine (3.4%) showed loss of two or more by immunohistochemistry. Interestingly, only seven (2.5%) cases demonstrated paired loss of hMLH1 and hPMS2 or hMSH2 and hMSH6, as is conventionally described in colon cancer in hereditary non-polyposis colon cancer (HNPCC). Another interesting finding is the proportion of tongue (1%) SCC demonstrating aberrant loss of MMR is significantly less than those of the gingiva (13%). While an explanation for this phenomenon is not readily available in the literature, Wang *et al.* report a similar low incidence of MMR dysregulation in a study predominantly including tongue SCC.[16] Studies examining the MMR phenomena in oSCC often report conflicting results due to limited sample size, heterogeneous cohorts of head and neck squamous cell carcinoma (HNSCC) which include cutaneous and oropharyngeal SCC, and differing techniques of MMR system analysis, ranging from immunohistochemistry to polymerase chain reaction (PCR) techniques.[14] The low incidence of MMR protein loss in our study is in contrast to the published literature on malignant oral lesions. This reflects the variations in cohort selection, as the current cases do not include oropharyngeal cancers, and the MMR detection methods and reporting criteria used by different studies. For example, Wagner and colleagues used digital evaluation of percentage of tumour nuclei stained to develop cut-off points for 'high' and 'low' protein expression, as opposed to the dichotomous approach ('intact' or 'loss') that is commonly used in routine clinical practice for colorectal and gynaecological malignancies.[30] Immunohistochemical evaluation of MMR can show interobserver variability due to misinterpretation of cytoplasmic staining as intact nuclear MMR expression, or misconstruing heterogeneous or weak staining as loss of MMR expression as demonstrated by Markow *et al.*[37] This is particularly true in the context of squamous cell carcinoma where there can be aberrant adsorption of the immunostain on keratin debris. More importantly, the presence of a germline or somatic mutation in MMR genes can still result in the production of a non-functional protein with retained antigenicity to immunohistochemical antibodies, producing a false positive result.[38] Interestingly, the documented rates of MSI in HNSCC range widely from 2 to 60%, analogous to MMR IHC.[39] Whilst Wang and colleagues in 2001 showed MSI to be more prevalent in younger patients with decreased exposure to traditional risk factors as compared to older patients, this has not been borne out in more recent studies, including an MSI study of 91 tumours which showed no correlation with age.[16,39] These variable results can be attributed to divergent definitions of MSI in PCR testing and a lack of an established MSI panel for HNSCC.[39,40]

The mechanisms for aberrant MMR expression in oSCC vary across the literature. Most studies to date have examined promoter hypermethylation and subsequent epigenetic silencing as the mechanism of oral carcinogenesis, with conflicting results. Wang *et al.*[16] assessed the rate of hMLH1 and hMSH2 promoter region hypermethylation and found no cases to support this in HNSCC. By contrast, promoter region hypermethylation of hMLH1 and hMSH2 has been shown by both Czerninski *et al.*[20] (50%, n=28) and Gonzalez-Ramirez *et al.*[21] (76%, n=50) in oral squamous cell carcinoma (oSCC) patients. Tobacco induced promoter region hypermethylation of MMR gene sites is reported to be a possible aetiological process driving mismatch dysregulation in HNSCC,[25] with Sengupta *et al.*[18] demonstrating hypermethylation of hMLH1 and hMSH2 to be associated with tobacco smoking. *BRAF* mutations have also been considered as a possible cause of promoter hypermethylation and subsequent silencing of MLH1. However, *BRAF* mutations do not occur frequently in non-colorectal malignancies, including urothelial and

Table 2 Cohort clinicopathological features and association with MMR status

Variable	Normal MMR (*n*=264)	Abnormal MMR (*n*=21)	*p* value
Age, mean years (SD)	63.0 (13.7)	65.4 (12.1)	0.45
Gender			
Female	111 (41%)	8 (38%)	0.82
Male	153 (58%)	13 (62%)	
Smoking status			
Smoker	160 (61%)	14 (67%)	0.65
Non-smoker	103 (39%)	7 (33%)	
Alcohol status			
Drinker	112 (83%)	11 (85%)	>0.99
Non-drinker	23 (17%)	2 (15%)	
Mucosal site			
Tongue	108	5	
Floor of mouth	64	7	
Gingiva	39	6	
Buccal mucosa	27	2	
Retromolar area	16	1	
Hard palate	7	0	
Differentiation			
Well	24 (9%)	3 (14%)	0.18
Moderate	167 (65%)	16 (76%)	
Poor	66 (26%)	2 (10%)	
Depth of involvement			
<5 mm	34 (15%)	6 (30%)	0.27
5–10 mm	61 (27%)	4 (20%)	
>10 m	128 (57%)	10 (50%)	
Tumour diameter			
Median (IQR)	30.0 (21.0–37.5)	25.0 (15.0–32.5)	0.11
Tumour margin			
Clear	30 (11%)	3 (14%)	0.47
Close	179 (68%)	12 (57%)	
Involved	53 (20%)	6 (29%)	
T-stage			
T1	44 (16%)	9 (43%)	<0.001
T2	106 (40%)		
T3	21 (8%)		
T4	92 (35%)	12 (57%)	
N-stage			
N0	138 (52%)	10 (48%)	0.85
N1	31 (12%)	3 (14%)	
N2/3	94 (36%)	8 (38%)	
Perineural invasion	118 (45%)	7 (33%)	0.37
Lymphovascular invasion	68 (26%)	6 (29%)	0.80
Bone invasion	84 (32%)	11 (52%)	0.05
Treatment			
Surgery	130 (49%)	6 (29%)	0.04
Surgery + radiation	100 (38%)	14 (67%)	
Surgery + radiation + chemotherapy	34 (13%)	1 (5%)	
Second malignancy			
Present	36 (30%)	5 (42%)	0.51
Information NA	144	4	
Number of malignancies			
1	30 (25%)	2 (17%)	0.05
2	3 (2%)	1 (8%)	
3	1 (1%)	2 (17%)	
4	1 (1%)		
Malignancy site			
Breast	6	1	
Lung	6	1	
Prostate	6		
Colonic polyp	5		
Gastrointestinal	4	1	
Haematological	4		
Other	2	4	
HNSCC	9	3	

HNSCC, head and neck squamous cell carcinoma; IQR, interquartile range; MMR, mismatch repair; NA, not available; SD, standard deviation.

Table 3 Frequency of MMR protein loss

Patient no.	MutSα		MutLα	
	hMSH2	hMSH6	hMLH1	hPMS2
1	L	L	L	L
2	L	L	L	L
3	L	L	L	L
4			L	L
5			L	L
6			L	L
7	L		L	L
8		L		L
9		L	L	
10	L			
11		L		
12		L		
13		L		
14		L		
15		L		
16			L	
17			L	
18		L		
19			L	
20	L			
21			L	
Total	6	11	12	8

L, loss of MMR protein; MMR, mismatch repair.

endometrial carcinoma.[41,42] A similar observation has been made in oral squamous cell carcinoma, where only one of 66 cases of oral tongue SCC harboured a *BRAF* mutation.[43] In contrast to promoter hypermethylation studies, there is a paucity of literature in HNSCC evaluating the role of mutations within the MMR genes themselves, with two studies to date detailing increased incidences of single nucleotide polymorphisms and deletions in MMR genes in HNSCC.[27,44]

We evaluated the history of metachronous malignancies in this cohort with a view to gaining an insight into the presence of a tumour predisposition phenotype. This is the first to identify a higher number of metachronous malignancies in MMR deficient patients with oSCC. This is not well characterised in the literature.[45] Interestingly, breast and lung cancer were the most common sites of metachronous malignancy amongst the MMR deficient patients in this cohort. Smoking is an established risk factor for lung malignancies. However, both lung and breast cancers do form part of the spectrum of Li-Fraumeni syndrome secondary to *TP53* gene mutations.[46] Helal Tel *et al.*[23] demonstrated increased p53 and reduced hMSH2 expression in oral SCC, as opposed to oral dysplasia where the opposite was found. This indicates that dysregulation of MMR pathways may play a role later in the development and progression of oral cancer, rather than as an early causative phenomenon. It may also explain why only 2.5% of the cases in this cohort demonstrated paired loss of hMLH1 and hPMS2 or hMSH2 and hMSH6 as expected in HNPCC related malignancies.

This is the first study to demonstrate a significant relationship described between MMR deficiency with advanced T category and bony invasion. Cellular mismatch repair dysfunction generates mutations at coding microsatellites. This leads to length changes in microsatellites, developing frameshift mutations and thus causing functional inactivation

Table 4 Association of baseline patient demographics and risk factors with survival outcomes

	Overall survival		Disease-free survival		Disease-specific survival	
	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
Age (<50 vs ≥50)	2.09 (1.22–3.60)	0.008	2.15 (1.29–3.57)	0.003	1.12 (0.55–2.28)	0.76
Gender (female vs male)	0.95 (0.67–1.35)	0.77	0.83 (0.60–1.14)	0.26	1.13 (0.65–1.95)	0.66
Treatment						
Surgery + radiotherapy	1.47 (1.01–2.13)	0.12	1.52 (1.08–2.14)	0.05	1.83 (0.99–3.37)	0.01
Surgery + radiotherapy + chemotherapy	1.38 (0.78–2.47)		1.48 (0.88–2.47)		3.10 (1.47–6.53)	
Risk factors						
MMR status (normal vs deficient)	1.36 (0.75–2.46)	0.32	1.31 (0.76–2.28)	0.33	0.43 (0.11–1.70)	0.23
Smoker	0.88 (0.62–1.25)	0.47	0.89 (0.65–1.24)	0.50	0.95 (0.55–1.63)	0.85
Alcohol drinker	1.21 (0.59–2.46)	0.60	1.19 (0.73–1.95)	0.49	1.21 (0.43–3.40)	0.72

CI, confidence interval; MMR, mismatch repair.

Table 5 Mismatch repair pathway in mucosal head and neck cancer in the literature

Study	MMR	Cohort	MMR loss	Technique	Conclusion
Current study	MSH2 MSH6 MLH1 PMS2	285 oSCC	21 (7.4%)	IHC	MMR loss associated with higher T-stage disease; low incidence of MMR loss
Lo Muzio *et al.*, 1999[15]	MSH2 MLH1	20 oSCC	1 (5%)	IHC	Absent staining may indicate potential phenotype mutator for oSCC
Wang *et al.*, 2001[16]	MSH2 MLH1	57 oSCC	0 (0%)	Promoter methylation IHC	MMR inactivation plays no role in MSI in oSCC tumours
Demokan *et al.*, 2006[17]	MSH2 MLH1	116 HNSCC	69 (59%)	Promoter methylation	Modifications in MSH2/MLH1 are significant in HNSCC
Sengupta *et al.*, 2007[18]	MSH2 MLH1	123 HNSCC	61 (50%)	Promoter methylation	Smokers are more susceptible to promoter methylation
Fernandes *et al.*, 2008[19]	MSH2 MLH1	46 oSCC	10 (22%)	IHC	Possible association of oSCC and HNPCC in tumours with both MMR proteins deficient
Czerninski *et al.*, 2009[20]	MSH2 MLH1	28 oSCC	14 (50%)	Promoter methylation	Hypermethylation may play a role in oSCC and developing multiple oSCC
Gonzalez-Ramirez *et al.*, 2011[21]	MLH1	50 oSCC	12 (24%) 38 (76%)	IHC promoter methylation	High frequency of MLH1 loss and in early stage (T1/T2) cancer
Tawfik *et al.*, 2011[22]	MLH1	49 oSCC	15 (30%) 14 (28%)	IHC promoter methylation	Promoter methylation is important in gene inactivation
Helal Tel *et al.*, 2012[23]	MSH2	70 oSCC	26 (37%)	IHC	Role of p53 and MMR interconnected in carcinogenesis of oSCC
Sarmento *et al.*, 2013[24]	MLH1 MSH2	40 Lip AC/SCC	–	IHC	Worsening dysplasia had decreasing index of expression
Jha *et al.*, 2013[25]	MLH1	242 oSCC	–	PCR-RFLP	hMLH1 −93 A>G polymorphism is related to oSCC
Wang *et al.*, 2013[26]	MSH3 MSH6 PMS1 PMS2	36 oSCC	18 (50%)	IHC	MSI positive tumours had decreased or no expression of PMS1
Nogueira *et al.*, 2013[27]	MLH1 MSH2 MSH3 EXO1	420 HNSCC	–	PCR-RFLP	Inherited polymorphisms in MLH1, MSH2, MSH3, EXO1 may predispose to oSCC
Jessri *et al.*, 2015[28]	MSH2 MSH6 MLH1 PMS2	45 oSCC	–	IHC	hMLH1, hPMS2, and hMSH2 expression was reduced, indicating a possible utility in diagnosis
Jessri *et al.*, 2015[29]	MSH2 MSH6 MLH1 PMS2	113 oSCC	–	IHC	Expression index reduced with worsening dysplasia
Wagner *et al.*, 2016[30]	MSH2 MSH6	115 oSCC	–	IHC	Overexpression of proteins was a poor prognostic marker

AC, actinic cheilitis; HNSCC, head and neck squamous cell cancer; IHC, immunohistochemistry; MMR, mismatch repair; mSCC, mucosal squamous cell carcinoma; MSI, microsatellite instability; OL, oral leukoplakia; oSCC, oral squamous cell carcinoma; PCR-RFLP, polymerase chain reaction restriction fragment length polymorphism.

of the proteins coded.[47,48] Transforming growth factor beta type II receptor (*TGFBR2*) is a key gene often mutated in 90% MMR deficient colorectal tumours, leading to dysfunctional transforming growth factor beta (TGF-β) signalling.[49] Pino *et al.*[49] showed MSI colorectal carcinoma cell lines undergoing epithelial-mesenchymal transition (EMT) in response to TGF-β signalling, even in the presence of wild-type *TGFBR2*. Bony invasion of tumours in oSCC has been hypothesised to result from promotion of osteoclastogenesis, a pathway which can be activated by induction of EMT.[50] Therefore, the higher T stage in our MMR deficient patients may be explained by an increased sensitivity to factors which encourage EMT and tumour progression, thus resulting in locally aggressive tumour behaviour in oSCC.

This finding has significant implications from the treatment perspective, particularly as pT4 oSCC may receive platinum based chemotherapy. An intact MMR system fosters sensitisation to cisplatin. Cisplatin produces DNA lesions that require a functioning mismatch mechanism to induce cell apoptosis in response to the DNA damage.[10] *In vitro* chemosensitivity testing of oral and oropharyngeal carcinoma found samples with hMSH2 expression were more susceptible to cisplatin than hMSH2 deficient tumours.[51] Further, Adachi *et al.*[11] discovered low hMLH1 protein expression in cisplatin-resistant HNSCC cell lines.

At present, MMR status in colorectal and endometrial cancers is being trialled as a surrogate marker of susceptibility to immunotherapy.[52,53] This is likely due to the characteristically increased neoantigen load of MMR deficient tumours caused by impaired DNA repair. This in turn elicits an intense host immune response, which provides a larger target for immune checkpoint inhibitors.[54] Immunotherapy results in HNSCC patients have shown some success, with programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) inhibitors showing increases in overall survival and treatment response rates.[55–58] However selection of patients for immunotherapy is made difficult by the lack of reproducibility in PD-L1 IHC cut-offs in HNSCC. One recent case report details a complete long term response despite a negative PD-L1 IHC result, in the setting of loss of PMS2 and MLH1 by immunohistochemistry and MSI-high status by PCR.[59] This, in addition to findings in other solid tumours, indicates that MMR status may play a role in the selection of HNSCC patients for immunotherapy. Further research is required to determine whether MMR dysregulation has a role in patient selection for adjuvant chemotherapy and immunotherapy in oSCC.

The primary strengths of the study lie in the sample size and exclusion of cases that may be attributed to HPV-related mechanisms and long term follow up. Utilising TMAs may be considered a limitation of this study; however, TMA applications have been extensively validated in biomarker studies in multiple malignancies.[31] Furthermore, two cores from the most cellular areas were utilised from each tumour as per the standard recommendations.[37] The evaluation of MMR IHC has many pitfalls as described by Markow *et al.*[37] This study has used clinically validated staining protocols, external and internal controls and the staining has been evaluated by two pathologists. The retrospective nature of the study introduces biases in data collection of risk factor and a reliable history of metachronous malignancy history could only be obtained in half of the cohort.

CONCLUSION

The findings of our study indicate that the incidence of loss of immunohistochemical expression in oSCC is low and is of limited utility in detecting patients at increased risk of developing other malignancies. However, immunohistochemical testing for MMR proteins may be useful in patients with high stage disease being considered for chemotherapy.

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