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DNA ploidy and cell cycle protein expression in oral squamous cell carcinomas with and without lymph node metastases

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Background: Oral squamous cell carcinoma (OSCC) is the most frequently occurring malignant tumour in the oral cavity. OSCC arises because of multiple genetic alterations. Cell cycle aberrations and aneuploidy are reportedly among the main characteristics of cancer cells and are associated with aggressive growth and poor prognosis.

Methods: The study sample included 47 non-metastasised (NM) and 39 metastasised (M) primary OSCC, with matched positive cervical lymph nodes (LN) and 17 normal oral mucosa (NOM) samples. Tissue microarrays (TMAs) were prepared with a minimum of three cores from each case. TMA sections were cut and immunostained with MCM2, Ki-67, geminin and cyclin D1 antibodies. DNA image analysis was performed on the whole tissue section before TMAs were created.

Results: The results revealed that there were no differences in cell cycle protein expression in different areas of the tumours or between the metastatic and non-metastatic carcinomas. None of the cell cycle proteins showed significant differences between the lymph node metastasis and the primary OSCC, except for Ki-67. Geminin/Ki-67 ratio showed significant difference between metastatic and non-metastatic tumours. Aneuploidy was detected in all (100%) cases of OSCC. Similarly, all lymph node samples (39 cases) were aneuploid.

Conclusion: The results suggest that although there was dysregulation of cell cycle regulatory proteins, only Ki-67 and the MCM2/Ki-67 and geminin/Ki-67 ratios may have prognostic significance in oral cancer. DNA ploidy alone was not specific and may not be a good tool to evaluate prognosis or metastatic progression in oral cavity carcinomas.

Introduction

Squamous cell carcinoma comprises more than 90% of all malignancies in the oral cavity (1) and are characterised by a high rate of recurrence and metastasis to regional lymph nodes, with a 50% mortality rate (2). Nodal metastasis is considered to be a significant prognostic indicator (3).

Several studies have reported the clinical utility of cell cycle proteins as a diagnostic predictor of oral carcinoma transformation (4, 5). However, few studies have evaluated the regulatory cell cycle proteins in primary lesions along with their lymph node metastases (6, 7)

Oral squamous cell carcinoma (OSCC) arises as a consequence of multiple genetic alterations, which may cause cell dysregulation including alterations in cell cycle proteins, which contribute to the development and progression of lesions. Therefore, understanding of the biologic basis of these alterations is essential for proper diagnostic and prognostic evaluation.

Among the cell cycle regulators, minichromosome maintenance proteins (MCM2-7) are required for DNA replication and cell cycle initiation (8). MCM2 in particular has been proposed as a sensitive prognostic marker that may indicate the presence of an increased growth fraction in malignant lesions. The MCM2/Ki-67 ratio has been used as a prognostic parameter to estimate the population of cells that are licensed to proliferate (4). A high ratio indicates that many cells are in cycle, and could indicate a poor prognosis (9). Geminin is a proliferation marker expressed only during the S-G2-M transition. It is believed to regulate cell cycle initiation by preventing the second pre-RC assembly once replication has taken place (10). The geminin/Ki-67 ratio estimates the relative length of G1, where a short G1 phase will approximate to a ratio of 1, cells with a prolonged G1 will approximate to a ratio of 0 (11). A higher ratio, indicates a faster rate of cell division, and may be associated with a worse prognosis (4). Analysis of these biomarkers may provide prognostic information in OSCC and may help to predict which lesions are most likely to metastasise.

Aneuploidy, as a measure of abnormal nuclear DNA content, is known to be an indicator of numerical chromosomal and DNA aberrations, and is associated with a high risk of recurrence (12, 13). In addition, several studies have underlined biomarkers associated with aneuploidy in oral epithelial dysplasia with risk of malignant transformation (14-16). However, the prognostic importance of DNA ploidy in oral cancer remains uncertain.

DNA image cytometry (ICM) is widely used as a diagnostic and prognostic method for quantification of DNA content by means of integrated optical density (IOD). Furthermore, ICM provides two established parameters that may be used as quantitative measures of DNA alterations: the DNA index (DI) and the percentage of cells that exceed 5c (5cER) (17).

The purpose of this study was to evaluate cell cycle proteins (MCM2, Ki-67, geminin and cyclin D1) and DNA ploidy in metastatic and non-metastatic OSCC and to determine their potential as diagnostic or prognostic markers.

Materials and methods

Tissue Specimens

The pathology records in the Unit of Oral and Maxillofacial Pathology, School of Clinical Dentistry, University of Sheffield, were reviewed to identify cases of OSCC that had or had not metastasised. Metastatic cases were defined as histologically proven metastatic lesions in a cervical lymph node. Only cases that had been treated by primary surgery were included; cases that received

radiotherapy before surgical removal were excluded. Disease stage was defined in accordance with the TNM classification by the International Union Against Cancer (18). A total of 125 samples from 86 patients were included in this study, 47 cases of OSCC that had not metastasised (OSCC NM) and 39 cases that had metastasised (OSCC M), as well as the 39 matched positive lymph node lesions (OSCC LN). Seventeen cases of normal oral mucosa (NOM) that histologically looked normal were used as a control group. Clinical features for all studied samples are summarised in Table 1. Ethical approval for the project was obtained from South Sheffield Research Ethics Committee (08/S0709/70).

Immunohistochemical staining

Tissue microarrays (TMA) of specimens were constructed using a Beecher manual micro-array system (The L.S. Starrett Company, USA). For each sample a haematoxylin and eosin-stained section was examined and representative areas of the tumour were selected and marked on the glass slide using a cytology slide marker (Nikon). Care was taken to select representative areas of tumour with minimal stroma, and avoiding areas of necrosis or ulcer. Three areas were sampled for each OSCC primary tumour (surface, middle and advancing front) and whenever possible three cores were taken from each of those three areas, giving a maximum of nine TMA cores per tumour.

Four-micrometre (4μm) sections of the TMA paraffin blocks were cut and mounted on coated slides (Menzel Gläser, Germany). Sections were dewaxed and rehydrated in absolute alcohol. To inhibit endogenous peroxidase activity, sections were treated with freshly prepared hydrogen peroxide in methanol (3% Met OH) for 30 minutes at room temperature. Then, a heat-mediated antigen retrieval protocol was carried out using citrate buffer (pH 6.0) in a steamer, for 30 minutes, for antibodies against MCM2, Ki-67 and geminin. For antibodies to cyclin D1 Tris-EDTA buffer (pH 9.0) was used for 30 minutes. Sections were incubated for 60 minutes, with normal horse serum for mouse monoclonal antibodies, or goat serum for rabbit polyclonal antibodies. Primary antibodies were diluted and incubated at 4°C overnight with anti-MCM2 (Sigma, 1:1000), anti-Ki-67(MIB-1, Dako, 1:75), anti-geminin (ab12147, Abcam, 1:300) and anti-cyclin D1 (EP12, Dako, 1:100). The visualisation system was the Vectastain ABC standard kit (Vector laboratories, US) that includes incubation with secondary biotinylated antibody and with an appropriate substrate for 30 minutes each at room temperature. Positive staining was detected after 6 minutes incubation with Vector Novared (Vector Laboratories, US). Finally, the slides were rinsed twice with distilled water and then counterstained with Mayer`s haematoxylin before being dehydrated and mounted with low viscosity DPX mounting media.

In addition, all cases were stained with $p16^{INK4A}$ (2D9A12, Abcam,1:500) to exclude human papilloma virus (HPV) associated lesions. Sections from a known HPV positive oropharyngeal carcinoma were used as a positive control to ensure antibody specificity for $p16^{INK4A}$. Immunostaining of $p16^{INK4A}$ was considered positive if there was strong and diffuse nuclear and cytoplasmic staining in more than 70% of tumour cells (19, 20).

Expression of each protein was quantified by cell counting. Ten high power fields (×400 magnification) were identified using a stratified random sampling method. An eyepiece graticule was used to delineate the counting area. A cumulative mean technique was used to determine the minimum number of cells that must be counted. For each case, a total of 800 to1500 nuclei were counted and a labelling index (LI) was calculated as follows:

LI = (total number of positive cells / total number of cells counted per case) × 100

DNA image cytometry

DNA content was evaluated using the Automated Cellular Imaging System, ACIS III (Dako, Glostrup, Denmark) using whole tissue sections. Briefly, 7µm paraffin sections were cut and slides were stained with Feulgen-Schiff stain, following the manufacturer`s instructions (ScyTek Laboratories Kit, USA). The ACIS system automatically scans the slides at ×400 to produce a virtual microscopy image. Using a pre-set algorithm the system then identifies abnormal nuclei. The operator then reviews the image to select areas of tumour for analysis and to confirm visually that the selected cells meet the criteria for control cells or malignant cells. Unwanted cells, for example, incomplete sections of nuclei, overlapping nuclei or obvious cell debris can be selected and rejected. For each case, at least 300 qualified malignant epithelial nuclei and approximately 50 control nuclei (lymphocytes) in the same tissue were selected. The optical dentisty of these nuclei was then measured by the system.

Criteria for classification of DNA histograms

High fidelity DNA histograms were evaluated according to the criteria described in previous studies (21-23). Ploidy was classified according to the value of the DNA optical index (DI) as follows: normal (diploid) (DI = 0.9–1.10), mild aneuploid (DI = 1.11–1.30), moderate aneuploid (DI = 1.31–1.80) and severe aneuploid (DI = >1.81). In the case of two peaks, the DI of the most prominent peak was considered.

Statistical analysis

Statistical analyses were undertaken using SPSS software (Version 21.0). ANOVA and *t*-tests were used to compare the mean LI of MCM2, Ki-67, geminin and cyclin D1 protein between groups (NOM, NM, M). Paired *t*-tests were used to evaluate differences in protein expression between primary OSCC and their matched LN metastases. Non-parametric tests were used to compare the DI, 5cER and ploidy status between groups. Spearman correlation tests were used to determine whether primary lesions with metastases and OSCC differentiation correlated with ploidy status. A p-value less than 0.05 was considered statistically significant. The general linear model (GLM) of repeated measures analyses was used to evaluate the differences in the LI of all proteins (MCM2, Ki-67, geminin and cyclin D1) in every group.

RESULTS

The clinical features of the 86 cases are summarised in Table 1. The mean age of NOM was 51.6 years (Standard deviation (SD) of 15.7). In the OSCC NM group the mean age was 63.86 years (SD = 12.6) and was 61.7 (SD = 12.55) in the OSCC M group. The primary tumours (OSCC NM and M) were histopathologically graded according to the WHO criteria (24). Of the 47 OSCC NM samples, 21 (45%) were well differentiated, 20 (43%) moderately differentiated and 6 (13%) poorly differentiated. In the samples that metastasised, 13 (33%) were well differentiated, 20 (51%) moderately differentiated and 6 (15%) poorly differentiated. There was no difference in differentiation between the two groups. In the metastatic node samples (OSCC LN), 15 (38%) were well differentiated, 21 (54%) were moderately differentiated and 3 (8%) were poorly differentiated. The single most common site for the tumours was the anterior (mobile) tongue.

All samples in the OSCC M group were stage III or IV, with the majority (69%) in stage IV, whereas all the OSCC NM cases were stage III or less with only 36% stage III (Table 1). In 36 of the metastatic (OSCC LN) lesions, the presence or absence of extracapsular spread (ECS) was recorded in the pathology records. The results showed that 16 out of the 36 cases (44%) showed ECS, while 20 (56%) showed no spread.

The $p16^{INK4A}$ staining confirmed that all cases (NOM, OSCC M, OSCC NM and OSCC LN) were negative and therefore this cohort did not include any HPV associated lesions.

Expression of cell cycle proteins

Expression of cell cycle proteins as determined by LI is shown in Table 2. The NOM samples showed positive nuclear staining for MCM2, Ki-67, geminin and cyclin D1 mainly in the basal cell layers. The NOM group had the lowest expression of MCM2, Ki-67 and geminin when compared with OSCC M and OSCC NM groups.

In the carcinoma samples, all the cell cycle proteins measured showed higher expression in the suprabasal, as well as the basal layers (Figures 1-2). There were no differences in expression for any of the measured markers between the different areas of the carcinomas (data not shown). Therefore, the LI data from each of the three areas was aggregated to give a single LI for each case (Table 2).

In all samples, expression of MCM2 was significantly higher than Ki-67 or geminin (GLM, $p<0.001$) (Table 2). MCM2 was also significantly higher in OSCC M (73.58) compared to NOM (60.09) ($p=0.007$, ANOVA), but there was no difference between metastatic (OSCC M) and non-metastatic (OSCC NM) lesions. Similarly, there were no significant differences between OSCC M and OSCC NM for Ki-67, geminin or cyclin D1 expression.

The geminin/Ki-67 ratio was significantly increased in carcinomas compared to NOM, but was also significantly increased in metastatic lesions (OSCC M; 0.68) compared to non-metastatic lesions (OSCC NM; 0.66) ($p = 0.02$, *t*-test). There were no differences in the MCM2/Ki-67 ratios.

Incidence and severity of aneuploidy

The DNA ploidy data is summarised in Table 3. There were no significant differences in ploidy status between OSCC M and OSCC NM, nor any correlation with tumour grade or stage (data not shown). The ploidy status of primary lesions and their corresponding metastases were compared. In 23 cases (59%), the degree of aneuploidy was the same in both lesions. In 8 of the 39 cases (21%) there was more severe aneuploidy in the lymph node metastases than in the primary lesion, and in a further 8 cases the changes were less severe. The ploidy parameters, DI and 5cER values, were significantly higher in the nodal metastases (OSCC LN) than in the primary carcinomas (OSCC M) ($p = 0.05$ and 0.01 respectively) (Table 3).

Discussion

MCM2 expression increased from normal through OSCC NM to OSCC M. The lower expression in NOM demonstrates that cells are in the G0 (not proliferating) or in G0–G1 phase. This suggests that normal epithelial cells have a low and organised proliferation rate but with constant proliferative ability. These results are in line with previous reports (4, 23). The geminin/Ki-67 ratio can potentially estimate the length of G1. Cells with an increased geminin/Ki-67 ratio have a shorter G1 phase and a high rate of cell proliferation. The geminin/Ki-67, but not MCM2/Ki-67 ratio for OSCC M increased significantly when compared with OSCC NM samples. The geminin/Ki-67 ratio has been proposed as a prognostic marker in oligodendroglial tumours (11), and the significant differences in the geminin/Ki-67 ratio between OSCC NM and OSCC M, suggest an accelerated G1 phase in metastatic oral tumours, which may provide a potential parameter for prognostic assessment of OSCC.

There was no difference in the cell cycle regulatory proteins between primary and matched lymph node metastases, except for Ki-67, with higher expression in primary tumours. Higher expression in the primary lesions than the lymph nodes may reflect a lower proliferative capacity of metastatic tumour cells (25). More interestingly, geminin/Ki-67 and MCM2/Ki-67 ratios were lower in the nodal metastases than in the primary lesions, and these differences were statistically significant. Further investigation will confirm whether these ratios are reliable markers of metastasis in OSCC.

In contrast, MCM2 expression was greater in the nodal metastases, but with no statistical significance. Low Ki-67 and high MCM2in the nodal metastases may suggest that a large population of metastatic cells are in G0 phase, but have proliferative potential (licensed). High MCM2 may reflect a cell proliferation capacity of OSCC in nodal metastases and supports the idea that metastatic lesions may be better differentiated (26). However, no study has yet explored the proliferative activity of OSCC in lymph nodes.

All OSCC cases (NM & M) were aneuploid (100%). This result differs from previous studies which have recorded a lower aneuploidy incidence in OSCC (22, 27). Such variation may be attributable to differences in methodology used in each study. In the present study, ICM was used on whole tissue sections, which has the advantage that tumour areas can be accurately selected, ensuring representative sampling and exclusion of non-tumour cells. Additionally, DNA ploidy is known to be heterogeneous within OSCC and the rate of abnormal content (aneuploidy) is higher when multiple samples are analysed in each tumour (28, 29). Likewise, all lymph node metastasis lesions showed DNA abnormalities with varying degrees of severity. DI and 5cER parameters were significantly higher in the metastatic lesions than primary carcinomas. This suggests aberrant or increased DNA

content in metastatic tumour cells. This could also indicate that cells with high levels of aneuploidy are more likely to metastasise and may be a characteristic of progressive disease.

In contrast, other studies did not find any association between high aneuploid incidence and high metastasis rates (30). However, confirmatory studies on quantitative evaluation of DI and 5cER are needed if these parameters are to have any prognostic value.

Alterations in cell cycle proteins, including licensing proteins and regulators, may contribute to the development and progression of OSCC. MCM2 showed an increase from normal through OSCC NM to OSCC M, indicating the presence of a high number of cells licensed to proliferate. This result suggests that MCM2 may provide useful information about the growth fraction in normal and malignant cells. However, MCM2 did not show any significant difference in OSCC with or without metastasis. In addition, the geminin/Ki-67, but not MCM2/Ki-67 ratio for OSCC M was significantly associated with nodal metastases. No significant associations between MCM2, Ki-67, geminin and cyclin D1 and the TNM stages, lymph node metastases or differentiation were found. All cases showed abnormal DNA content, so DNA ploidy alone may not be a good diagnostic tool to evaluate OSCC progression in the oral cavity. Although, abnormal DNA content may be a necessary feature of OSCC, it is not specific to progressive lesions.

Conflict of Interest Statement

The authors have no financial interest to declare in relation to the content of this article.

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Figure Legends

Figure 1:

Representative immuno-staining of MCM2 (A), Ki-67 (B), geminin (C), and cyclin D1 (D) proteins in samples of OSCC that did not metastasise (OSCC NM). All images are at ×200 magnification.

Figure 2:

Representative immuno-staining of MCM2 (A), Ki-67 (B), geminin (C), and cyclin D1 (D) proteins in samples of OSCC that did metastasise (OSCC M). All images are at ×200 magnification.

Table 1: Clinical features for all studied samples.

Clinical features	Number (%) in each group NOM (n = 17)	OSCC NM (n = 47)	OSCC M (n = 39)
Mean age (years)	51.55 ± 15.71	63.86 ± 12.58	61.67 ± 12.55
Gender			
Male	8 (47)	32 (70)	26 (67)
Female	9 (53)	15 (30)	13 (33)
Tumour grade			
Well	--	21 (45)	13 (33)
Moderate	-	20 (43)	20 (51)
Poor	-	6 (13)	6 (15)
Tumour stage (TNM)			
I		15 (32)	0
II		15 (32)	0
III		17 (36)	8 (21)
IV		0	27 (69)
NA		-	4 (10)
Total		47	39
Tumour size stage			
pT1		15 (32)	7 (18)
pT2		15 (32)	10 (26)
pT3		17 (36)	4 (10)
pT4		0	18 (46)
Total		47	39

NOM = Normal oral mucosa
OSCC M = Oral squamous carcinoma that had metastases
OSCC NM = Oral squamous carcinoma that did not metastasise
Percentages (%) in brackets

Table 2: Mean and standard deviation of MCM2, Ki-67, geminin and cyclin D1 labelling index (LI) values, along with MCM2/Ki-67 and geminin/Ki-67 ratios for all groups

	Labelling index (LI) **Mean (Standard deviation)**						
	MCM2	Ki-67	Geminin	Cyclin D1	Stat. GLM	MCM2/Ki-67 ratio	Geminin/Ki-67 ratio
NOM (n = 17)	60.09 (16.46)	47.16 (21.43)	27.34 (5.97)	58.99 (6.55)	p < 0.001	1.55 (0.67)	0.58 (0.23)
OSCC NM (n = 47)	69.96 (16.28)	64.61 (28.31)	34.23 (14.11)	55.35 (17.96)	p < 0.001	1.35 (1.00)	0.66 (0.27)
OSCC M (n = 39)	73.58 (16.12)	54.23 (21.39)	31.16 (15.94)	56.84 (12.97)	p < 0.001	1.58 (0.84)	0.68 (0.30)
ANOVA test	**p = 0.007**	NS	NS	NS		NS	NS
t-test (OSCC M vs OSCC NM)	*NS	*NS	*NS	*NS		*NS	**p* = 0.02**
OSCC LN (n = 39)	79.88 (12.66)	49.66 (36.04)	20.75 (21.90)	59.20 (20.81)	p < 0.001	1.27 (0.43)	0.31 (0.30)
Paired *t*-test	p = 0.84	**p = 0.03**	p = 0.21	p = 0.27		**p = 0.05**	**p = 0.01**

NOM = Normal oral mucosa, OSCC NM = Oral squamous carcinoma that did not metastasise, OSCC M = Oral squamous carcinoma that had metastases, OSCC LN = Lymph node metastases, Stat GLM = General linear mode

NS = Not significant. Paired *t*-test between primary tumours (OSCC M) and their lymph node metastasis (OSCC LN)

Table 3: Incidence of DNA ploidy status, DI and 5cER in OSCC NM, OSCC M and OSCC LN

Groups	Mild aneuploid n (%)	Moderate aneuploid n (%)	Severe aneuploid n (%)	DI Median (25%-75%)	5cER Median (25%-75%)
OSCC NM (n = 47)	2 (4)	25 (53)	20 (43)	1.63 (1.44–1.91)	5.06 (2.60–10.79)
OSCC M (n = 39)	0	21 (54)	18 (46)	1.61 (1.50–1.79)	3.36 (1.00–7.80)
Chi-Square test		p = 0.5		p = 0.82	p = 0.17
OSCC LN (n = 39)	0	21 (54)	18 (46)	1.70 (1.5–2.20)	9.90 (0.83–15.70)
Wilcoxon Signed Rank Test		p = 0.2		**p = 0.05**	**p = 0.01**

OSCC NM = Oral squamous cell carcinoma that had not metastasis. OSCC M = Oral squamous cell carcinoma that had metastasis. OSCC LN = Lymph node metastasis. DI = DNA index. 5cER = Number of cells that exceed 5c