Microvessel Density in Different Grades of Oral Squamous Cell Carcinoma and its Relationship with Keratin Pearl

(Kepadatan Saluran Mikro dalam Gred Berbeza Karsinoma Sel Skuamus Mulut
dan Hubungannya dengan Mutiara Keratin)

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ABSTRACT

Angiogenesis has an important role in the invasion, metastasis and growth of tumors. Increased microvessel density (MVD) has been described in oral squamous cell carcinoma (OSCC) compared to oral dysplasia and normal oral tissue. A morphometric study was designed to evaluate the MVD and to evaluate the presence of any association between MVD and keratin pearl in the three histopathological grades of OSCC (as defined in Bryne's grading system). Forty-five samples of OSCC were graded into well differentiated (WDSCC), moderately differentiated (MDSCC) and poorly-differentiated (PDSCC) oral squamous cell carcinoma. Morphometric analysis showed that MVD was significantly lower in WDSCC compared to MDSCC ($p<0.001$) and PDSCC ($p<0.001$). The density in MDSCC was significant lower than PDSCC. The keratin pearl count was significantly higher in WDSCC compared to MDSCC ($p<0.001$) and PDSCC ($p<0.001$). Between MDSCC and PDSCC, the keratin pearl count was significantly higher for MDSCC ($p=0.001$). Pearson correlation test showed a significant negative correlation between keratin pearl and MVD ($r=-0.805$, $p<0.001$). The findings suggested that vascularity in OSCC is associated with its cellular differentiation and also associated with keratin pearl formation.

Keywords: Keratin pearl; microvessel density (MVD); oral squamous cell carcinoma (OSCC); tumour grading

ABSTRAK

Angiogenesis mempunyai peranan yang penting dalam penaklukan, perebakan dan juga pertumbuhan tumor. Peningkatan kepada kepadatan saluran mikro (MVD) telah digambarkan di dalam karsinoma sel skuamus mulut (OSCC) jika dibandingkan dengan displasia mulut dan tisu mulut biasa. Suatu kajian morfometri telah direka untuk mengkaji kepadatan saluran mikro (MVD) dan mengkaji kehadiran mana-mana yang berkaitan antara MVD dan mutiara keratin di dalam tiga gred histopatologi terhadap OSCC (seperti yang diterangkan di dalam sistem pengredan Bryne). Empat puluh lima sampel OSCC telah digredkan kepada perbezaan yang sangat baik (WDSCC), perbezaan yang sederhana (MDSCC) dan perbezaan yang kurang atau tiada (PDSCC) bagi karsinoma sel skuamus mulut. Analisis morfometri menunjukkan bahawa MVD adalah jauh lebih rendah di dalam WDSCC berbanding MDSCC ($p<0.001$) dan juga PDSCC ($p<0.001$). Manakala ketumpatan saluran mikro di dalam MDSCC adalah lebih rendah berbanding PDSCC. Kiraan mutiara keratin adalah lebih tinggi di dalam WDSCC jika dibandingkan dengan MDSCC ($p<0.001$) dan PDSCC ($p<0.001$). Antara MDSCC dan PDSCC, jumlah kiraan mutiara keratin adalah lebih tinggi di dalam MDSCC ($p=0.001$). Ujian korelasi Pearson menunjukkan hubungan negatif yang signifikan antara mutiara keratin dan MVD ($r=-0.805$, $p<0.001$). Hasil kajian menunjukkan bahawa saluran vaskular di dalam OSCC dapat dikaitkan dengan pembezaan sel dan pembentukan mutiara keratin.

Kata kunci: Karsinoma sel skuamus mulut (OSCC); kepadatan saluran mikro (MVD); mutiara keratin; pengredan; tumor

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cause of cancer related to death in the world (Warnakulasuriya 2009) and is the most frequent malignancy in the mouth, accounting for up to 90% of all oral malignant lesions (Warnakulasuriya 2009). Despite the advances in surgery and multimodal treatment regimes, the prognosis of OSCC remains relatively poor (Tannapfel & Weber 2001). Mortality in OSCC patients is often caused by tumour lymph node metastasis, rather than by the primary tumour (Tannapfel & Weber 2001).

In order to improve the prognosis of OSCC, early detection is crucial. The most commonly used and worldwide accepted grading system for cancer is the Tumor Node Metastasis (TNM) system (Deschler & Day 2008; Snehal & Jatin 2005). However, identifying the lymph node metastasis and distant metastasis with TNM system is difficult and often not very effective (Doshi et al. 2011). The TNM grading used in clinical practice only addresses the anatomical extend and does not provide information on the biological characteristic of cancer cells. Hence a multitude of multifactorial grading systems have been

developed (Doshi et al. 2011). Bryne's grading system that uses degree of keratinization, nuclear pleomorphism, pattern of invasion and lymphoplasmacytic infiltration as parameters showed significant relationship with lymph node metastasis (Bryne et al. 1992; Doshi et al. 2011).

Along with the aforementioned histological parameters, the role of angiogenesis in OSCC is important. Angiogenesis, the growth of capillary vessels, plays an important role in the metabolic functions of malignant tissues. Increased angiogenesis has been associated with neoplastic progression, metastasis in a number of malignancies (Kyzas et al. 2005; Linder et al. 2001; Tanigawa et al. 1997; Xiangming et al. 1998; Yoshij et al. 1996). Angiogenesis has been found to be significantly increased in OSCC tissue (Li et al. 2005). There is a possibility that angiogenesis can be incorporated to the existing grading systems. Therefore, we design a morphometric study to evaluate the microvessel density in Haematoxylin-Eosin (H&E) stained sections as H&E staining is commonly used in normal laboratory procedure.

Apart from angiogenesis, keratin pearl is one of the other histological parameters in the histopathological malignancy grading. Keratin pearls indicates the maximum limit of differentiation. We hypothesise that there may be a relation among keratin pearl formation, malignancy grading and microvessel density.

The objectives of this present study were to determine whether vascularity has a relation with differentiation in OSCC tissue and to assess the association between vascularity and keratin pearl in OSCC.

MATERIALS AND METHODS

SAMPLE SELECTION

A total of 45 histologically proven diagnoses of oral squamous cell carcinoma samples were retrieved from the archives of the histopathology laboratory from the International Medical University and from the Department of Oral Medicine and Maxillofacial Pathology in University of Dental Medicine in Yangon, Myanmar. The study protocol was approved by the ethics approval committee of the institutions. All lesions that were primary tumours arising intra-orally, e.g. tongue, floor of the mouth, cheek, gingiva and palate or retromolar trigone and treated with wide excision of growth were included. Tumours not arising from the oral cavity proper, e.g. vermillion border of the lip and pharyngeal complex were excluded. Tumours that involve overlying skin, mandibular bone, resection specimen following radio- or chemotherapy and recurrent tumours were also excluded.

TRAINING AND CALIBRATION

A separate group of sample comprising of ten OSCC was used for training and calibration in the grading of OSCC, microvessel density and keratin pearl counts with one-week intervals between examinations to assess and to achieve intraexaminer and interexaminer reliability coefficient.

HISTOPATHOLOGICAL GRADING OF SAMPLES

The samples in paraffin blocks were cut into sections and Haematoxylin-Eosin (H&E) staining was done. All H&E stained sections were re-examined microscopically and are then graded according to the four parameters advocated in the Bryne's grading system (Bryne et al. 1992) into the three histopathological grades: Well-differentiated (WDSCC); moderately-differentiated (MDSCC); and poorly-differentiated (PDSCC). Interexaminer and intraexaminer reliability coefficient was calculated during grading.

IDENTIFICATION AND COUNTING OF MICROVESSELS (MICROVESSEL DENSITY)

Any endothelial cells or endothelial cell clusters that was clearly separate from adjacent microvessels, tumour cells and other connective tissue elements was considered a single, countable microvessel as specified by Astekar et al. (2012). Vessel lumens, although usually present, were not necessary for a structure to be defined as a microvessel and red cells were not used to define a vessel lumen.

Microvessel density was defined as the number of vessels per slide. Three sites (the area most populated by blood vessels) within the slide of a section were selected and microvessels were counted under a light microscope (Nikon Eclipse 55i) with a 200-fold magnification. Each of the three sites was counted three times and the mean count is obtained.

IDENTIFICATION AND COUNTING OF KERATIN PEARLS

Keratin pearls are whorl-shaped accumulations of keratin usually present as pink, glassy, spherical masses within epithelial stroma that can be seen under light microscope. Keratin pearl count is defined as the number of keratin pearls per slide. Three sites from each slide were selected and counted under a light microscope (Nikon Eclipse 55i) with a 100-× magnification. Each of the three areas was counted three times and the mean count was obtained.

STATISTICAL ANALYSIS

In order to achieve a homogenous sample size, 30 sections were randomly chosen from the samples for each grade of OSCC. In order to test the differences concerning microvessel density (MVD) and keratin pearl count between the three grades of OSCC according to the Bryne's grading system, one way analysis of variance (ANOVA) followed by Tukey's test was used. Correlations between MVD and keratin pearl counts were assessed using the Pearson's correlation test. For all tests a p-value of less than 0.05 was considered to be of statistical significance. Data were presented as mean ± SEM.

RESULTS

HISTOPATHOLOGICAL GRADING OF OSCC

From a total of 45 samples, 17 cases was graded as WDSCC, 15 cases as MDSCC and 13 cases as PDSCC. Interexaminer

and intraexaminer reliability coefficient during grading was satisfactory (0.80 & 0.79, respectively). Thereafter, 30 sections from each grade of OSCC was chosen randomly to proceed with MVD and keratin pearl count.

MICROVESSEL DENSITY

Highest MVD was found in the PDSCC (30.60 ± 2.11/section) and lowest was found in WDSCC (11.83 ± 0.66/section) (Figures 1(a) & 2(a)). The MVD for MDSCC was 20.27 ± 0.88/section. The MVD for WDSCC was significantly lower than that of MDSCC and PDSCC ($p<0.001$, one-way ANOVA followed by Tukey's test). There was statistically significant difference between the MVD of MDSCC and PDSCC ($p<0.001$). Interexaminer and intraexaminer reliability coefficient were in satisfactory level (0.75 & 0.80, respectively).

KERATIN PEARL COUNT

The keratin pearl count was highest for WDSCC and lowest for PDSCC (7.69 ± 0.62/section and 0.58 ± 0.11/section, respectively) (Figure 1(b) & 2(b)). For MDSCC it was 2.18 ± 0.17/section. Significant difference was observed between the keratin pearl counts of WDSCC and MDSCC ($p<0.001$) and between WDSCC and PDSCC ($p<0.001$) (Figure 2(b)). The difference of keratin pearl counts between MDSCC and PDSCC was also statistically significant ($p=0.001$). Interexaminer and intraexaminer reliability coefficient were in very good level (0.90 & 0.80, respectively).

CORRELATION BETWEEN MICROVESSEL DENSITY AND KERATIN PEARL

The Pearson's correlation test showed a significant ($p<0.001$) negative relationship ($r=-0.805$) between the MVD and the keratin pearl count in OSCC (Figure 2(c)).

DISCUSSION

Our study showed that microvessel density changes with the grading in oral squamous cell carcinoma. The microvessel density was observed to be more when OSSC was less differentiated. A significant negative correlation was found between microvessel density and keratin pearl formation.

Cancer cells are able to resist inhibitory signals originated from the extracellular matrix and the surfaces of nearby cells that retard their growth and are able to initiate and stimulate their own growth (Hanahan & Weinberg 2000). For any neoplasm to be classified as malignant, these cancer cells should be able to multiply uncontrollably and indefinitely and possess the ability to invade local tissue and spread to distant sites (metastasis) (Hanahan & Weinberg 2000). Other than resisting their own programmed cell death (apoptosis), cancer cells obtain nutrients by stimulating the growth of new blood vessels (angiogenesis) (Hanahan & Weinberg 2000). Angiogenesis is the process of formation of new microvessels from the pre-existing vasculature. No solid tumour can probably grow more than 1-2 mm in volume, unless it can synthesize its own network of new microvessels (Astekar et al. 2012; Elpek et al. 2001; Macluskey et al. 2000). The role of angiogenesis in neoplasia has been receiving increasing attention in recent times since there is a potential for it to be used as an independent prognostic indicator for tumour progression and metastasis and also as a novel second target for anticancer therapy instead of direct tumour cell inhibition (Elpek et al. 2001; Macluskey et al. 2000). The most widely used method to quantify angiogenesis is microvessel density (MVD). A study done by Li et al. (2005) has found that MVD significantly increase in OSCC tissue.

Another well established and useful histological factor in the histopathological malignancy grading of invasive tumour is keratin pearl formation (Piffko &
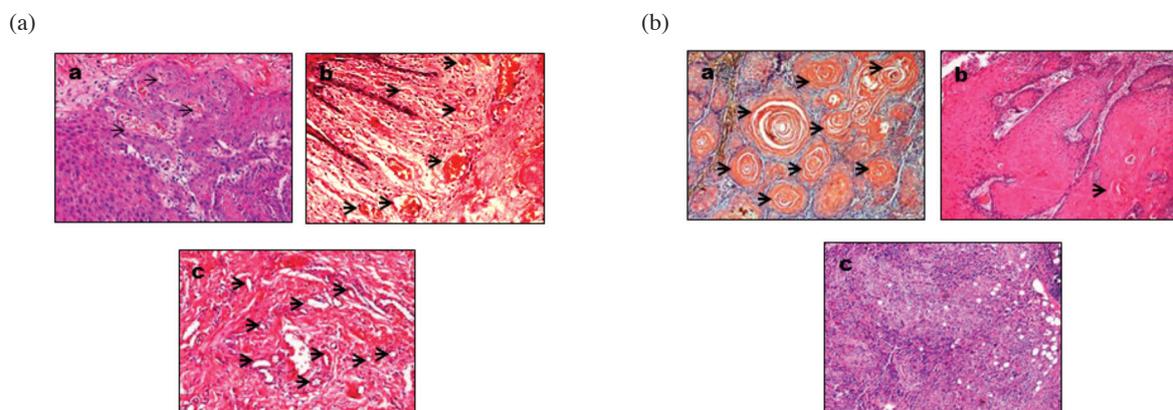
(a)

(b)

FIGURE 1(a). Tissue sections showing the microvessels in different histopathological grading of OSCC: Well-differentiated oral squamous cell carcinoma. (a) moderately-differentiated oral squamous cell carcinoma, (b) poorly-differentiated oral squamous cell carcinoma and (c) (H & E staining). Tissue sections showing the keratin pearls in different histopathological grading of OSCC: Well-differentiated oral squamous cell carcinoma. (a) moderately-differentiated oral squamous cell carcinoma, (b) poorly-differentiated oral squamous cell carcinoma and (c) (H & E staining)
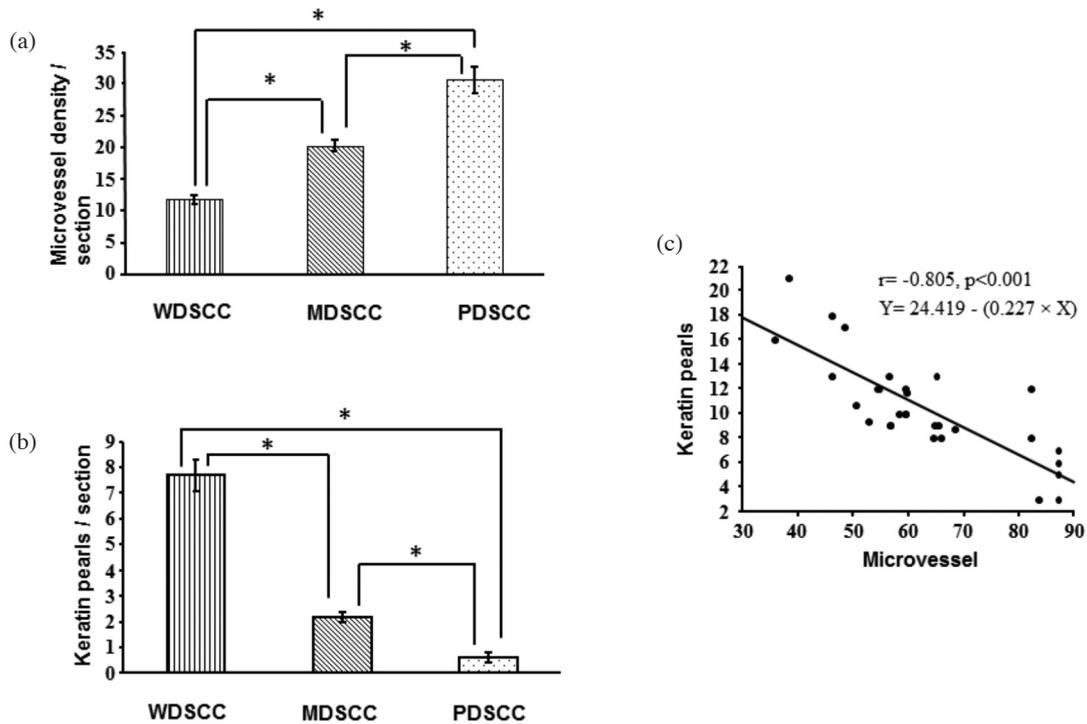
FIGURE 2. Comparison of microvessel density (a) and keratin pearl count (b) among different histopathological grading of OSCC. Note that microvessel density in WDSCC was significantly lower compared to PDSCC and MDSCC. On the other hand keratin pearl count in WDSCC was significantly higher compared to PDSCC and MDSCC. *p<0.05 (One way ANOVA followed by Tukey's test). Relationship between microvessel density and keratin pearl (c) (Pearson's correlation test)

Bankfalvi 1997). Keratin pearls represents the maximum limit of differentiation because they do not proliferate, further differentiate or invade the surrounding tissue. This is evidenced by a study done by Torres-Rendon et al. (2009) which shows a negative staining of keratin pearl with minichromosome maintenance protein 2 (Mcm2). This protein is necessary for the normal regulation of the cell cycle and to assess cell proliferation, Similarly, a histochemical analysis study by Chen et al. (2004) shows negative staining of keratin pearl with p73 and p63 which chiefly stain undifferentiated cells indicating keratin pearls are well differentiated. We can assume that keratin pearls are in non-progressive stage and therefore, they require very minimal amount of nutrition to survive. Our results of negative correlation of keratin pearl with MVD support this assumption. Cancer cells obtain nutrients from blood vessels for growth and maintenance. Our study has demonstrated that PDSCC has significantly higher vascularity compared to WDSCC, suggesting that in WDSCC, the source of nutrient is scarce. On the other hand the keratin pearl formation was significantly more in WDSCC compared to PDSCC. From the above findings, it can be postulated that in an attempt to balance out the effect of decreased in nutritional supply from the blood vessels, keratin pearls are formed in WDSCC.

Histopathological assessment of formalin-fixed, haematoxylin-eosin (H&E) stained biopsy tissue and surgical resection specimens remains the cornerstone of OSCC diagnosis and pathological staging in routine clinical practice. Our data suggested that morphometric analysis of microvessel density can be applied on any H&E stained slides without additional immunohistochemical staining for visualization of microvessels. However, supplementary studies are recommended to stain microvessels, immunohistochemically. Various factors such as vascular endothelial growth factor (VEGF), tryptase, fibroblast growth factor (FGF), tumour necrosis factor (TNF), interleukin (IL)-8, histamine and heparin were studied to identify microvessels, immunohistochemically in previous studies (Kyzas et al. 2005; Li et al. 2005; Linder et al. 2001; Lopez-Graniel et al. 2001).

The study has limitations. A limited number (forty-five) of cases were evaluated in this study. Research with a large number of samples may corroborate the findings. Labelling of microvessels immunohistochemically along with H & E staining would strengthen the findings.

CONCLUSION

Our study shows that vascularity has an inverse relationship with the degree of differentiation of OSSC. Furthermore, an inverse relationship found between vascularity and keratin pearl formation, which in turn reflects the differentiation of OSCC. The findings suggested the possibility of inclusion of vascularity as a histological parameter in OSCC grading and staging.

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