EFFICACY OF NEOADJUVANT CHEMOTHERAPY IN PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA IN A FEW SELECTED CENTRES IN KLANG VALLEY, MALAYSIA

NORUL MASHIRAH BINTI MOHD NOOR

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
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ABSTRACT

INTRODUCTION: Neoadjuvant chemotherapy (NAC) is purportedly advantageous in tumour reduction before definitive surgery is performed, with or without subsequent radiotherapy. Over the years, there have been conflicting results on the efficacy of NAC in advanced head and neck cancer, especially in oral cavity cancer. As the practice of NAC for oral squamous cell carcinoma (OSCC) is relatively new in Malaysia, there is a need to analyse the effectiveness of this treatment in the local settings for clinicians to consider NAC as a viable treatment option in future. This study aims to assess the effectiveness of NAC in patients with OSCC in Malaysia through histological assessment and podoplanin (PDPN) and VEGF expression. **METHODOLOGY**: For 14 OSCC patients treated with NAC, and subsequently surgery, tumour regression grading was performed using Tumour Regression Systems (Mandard-TRG, Ryan-TRG and AJCC-TRG) on all surgically excised tumour specimen slides. The corresponding pre-treatment biopsy specimens were investigated for Podoplanin and VEGF expression through immunohistochemistry. **RESULTS**: Only 2 out of 14 cases showed complete tumour regression while 9 cases had mild to absence of regression. AJCC-TRG was significantly correlated with larger tumour size (ypT) ($p=0.001$) and strongly associated with advanced stage, positive margin, positive lymph node metastasis, extracapsular spread and poorly differentiated tumour. High expression of PDPN was observed in cases with mild to absence of tumour regression while VEGF expression demonstrated no correlation with tumour regression. **CONCLUSION:** NAC did not offer added benefit as a treatment option for OSCC. AJCC-TRG system is recommended in grading tumour response in NAC-treated head and neck cancer and OSCC. PDPN showed potential as prognosticator for OSCC and as predictor of poor tumour response to NAC. VEGF was neither a good predictor of tumour response to NAC nor a promising prognosticator for OSCC.

Keywords: OSCC, Neoadjuvant chemotherapy, Tumour Regression Grading, Podoplanin, VEGF.

ABSTRAK

PENGENALAN: Kemoterapi neoadjuvant (NAC) mempunyai manfaat dalam pengecilan tumor sebelum pesakit kanser diberikan rawatan utama seperti pembedahan dengan atau tanpa rawatan radioterapi seterusnya. Selama beberapa tahun, terdapat pendapat yang bercanggah mengenai keberkesanan NAC dalam pesakit kanser kepala dan leher, terutamanya kanser mulut. Memandangkan amalan rawatan NAC untuk karsinoma sel squamous oral (OSCC) secara relatifnya agak baru di Malaysia, wujud keperluan untuk menganalisis keberkesanan rawatan ini sebagai salah satu pilihan rawatan yang sesuai digunakan dalam konteks tempatan. Kajian ini bertujuan untuk menilai keberkesanan rawatan NAC kepada pesakit OSCC di Malaysia melalui penilaian histologi, dan ekspresi Podoplanin (PDPN) dan juga VEGF. **KAEDAH**: Seramai 14 pesakit kanser OSCC yang telah dirawat dengan NAC dan kemudiannya dibedah telah dipilih. Slaid specimen tumor yang dibedah telah dianalisa untuk pengredan regresi tumor menggunakan sistem regresi tumor (Sistem Mandard, Ryan dan AJCC). Spesimen biopsi pra-rawatan NAC pesakit pula digunakan untuk penilaian pewarnaan PDPN dan VEGF melalui ujian immunohistokimia. **KEPUTUSAN**: Hanya 2 daripada 14 kes menunjukkan regresi tumor penuh manakala 9 kes pula menunjukkan sedikit ke tiada regresi. Sistem regresi tumor AJCC mempunyai korelasi signifikan terhadap saiz tumor yang lebih besar (ypT) (p=0.001) dan juga mempunyai tendensi hubungkait dengan peringkat tumor lanjutan, margin positif, metastasis ke nodul limfa, penyebaran luar kapsul dan tumor yang tidak membeza. Ekspresi PDPN yang berlebihan pula sangat berkorelasi dengan regresi tumor yang sedikit atau tiada, manakala ekspresi VEGF menunjukkan tiada korelasi terhadap regresi tumor. **KONKLUSI**: NAC tidak menawarkan faedah tambahan sebagai pilihan rawatan untuk OSCC. Sistem regresi tumor AJCC adalah dicadangkan bagi menilai tindak balas tumor dalam pesakit kanser kepala dan leher dan juga OSCC yang telah dirawat dengan NAC. PDPN mungkin adalah prognostikator bagi OSCC dan

ekspresi berlebihannya menunjukkan korelasi dengan greding regresi tumor. VEGF pula bukan merupakan factor predictor bagi tindak balas tumor terhadap NAC, dan bukan juga prognostikator berpotensi untuk OSCC.

Keywords: Kanser mulut, Kemoterapi neoajuvant, Pengredan regresi tumor, Podoplanin, VEGF.

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LIST OF SYMBOLS AND ABBREVIATIONS

AJCC		American Joint Committee on Cancer
ASR	:	Age standardized rate
ECS	:	Extracapsular spread
IARC	:	International Agency for Research on Cancer
IKN	:	Institute Kanser Negara/ National Cancer Institute
IRS	:	Immunoreactive score
LN	:	Lymph node
NAC	:	Neoadjuvant chemotherapy
NNK	:	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	:	N-nitrosonornicotine
OSCC	:	Oral squamous cell carcinoma
PAH	:	Polycyclic aromatic hydrocarbons
PCR	:	Pathologic complete response
PDPN	:	Podoplanin
PF	:	Cisplatin & 5-Fluorouracil
PlGF	:	Placenta Growth Factor
SCC	:	Squamous cell carcinoma
TPF	:	Cisplatin, 5-Fluorouracil & Docetaxel
TRG	:	Tumour regression grading
VEGF	:	Vascular endothelial growth factor
WHO	:	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Research background

Oral cavity cancer was the 16th most common cancer worldwide, and the 14th in South-East Asia region, contributing 2.0% of all cancer cases reported worldwide in 2018. Approximately 354,000 new cases were reported throughout the world in a year (GLOBOCAN, 2018; Bray et al., 2018). The most common type of oral cavity cancer is oral squamous cell carcinoma (OSCC). While the aetiology of oral cancer is multifactorial, it is known to be highly associated with habits of smoking, excessive alcohol consumption and betel quid usage. The latter has been established as the most important risk factor for population in the Asia continent with said habit such as in Malaysia, India and Taiwan (Zain & Ghazali, 2001).

Treatment of OSCC is mainly determined according to the stage of the cancer. For early stage OSCC, surgical approach is the principal single treatment modality that has been used (Omura, 2014). For patients presenting with advanced stage of OSCC, multimodality treatment is recommended, and surgery remains the first line treatment of choice, combining with radiotherapy afterwards (Wedemeyer et al., 2014)

Neoadjuvant chemotherapy (NAC) refers to the application of systemic chemotherapy before a malignant tumour is treated with surgery or radiation therapy. NAC, according to several studies, has potential benefits of tumour reduction, local control, decreased recurrence, decreased distant metastases, organ preservation in resectable tumours, less need of postoperative radiotherapy, and less need of destructive surgical procedures (Nanda & Mohiyuddin, 2015).

In Malaysia, to date, only a few centres in Klang Valley have started treating OSCC patients with neoadjuvant chemotherapy. These centres include Hospital Tengku Ampuan Rahimah, Hospital Kuala Lumpur, Hospital Serdang and Hospital Shah Alam where diagnosis, surgical management and follow up of the patients were conducted. NAC, or specifically the drug regimens are usually administered in the National Cancer Institute (IKN). There has not been any study done to investigate the efficacy of this treatment for patients with OSCC with regards to tumour response and local control rate in Malaysia.

1.2 Rationale of study

Neoadjuvant chemotherapy has been suggested to have main advantage in tumour reduction before definitive surgery with or without radiotherapy is performed. However, over the last 30 years, there have been conflicting results on NAC in randomized clinical trials in advanced head and neck cancer, especially in oral cavity cancer. Furthermore, as the practice of NAC for OSCC is relatively new in Malaysia, there is a need to analyse the effectiveness of this treatment in our local settings as reference when considering the NAC as a viable treatment option in future. This study will be using suggestive prognostic factors such as tumour response and expression of podoplanin and VEGF for this purpose.

1.3 Hypothesis

1) There is complete tumour regression in patients with OSCC treated with neoadjuvant chemotherapy.
2) There are positive correlations between regression grading and Podoplanin and VEGF expressions in patients with OSCC treated with neoadjuvant chemotherapy.

1.4 Aim

1) To assess the effectiveness of neoadjuvant therapy through histological assessment and podoplanin and VEGF expression.

1.5 Specific Objectives

1) To assess regression grading of patients with oral squamous cell carcinoma treated with neoadjuvant chemotherapy.
2) To investigate the association between regression grading and clinicopathological parameters in patients with oral squamous cell treated with neoadjuvant chemotherapy.
3) To assess the expressions of podoplanin and VEGF in biopsy specimens of patients with oral squamous cell carcinoma prior to treatment with neoadjuvant chemotherapy.
4) To investigate the association between regression grading and expressions of podoplanin and VEGF in patients with oral squamous cell carcinoma prior to treatment with neoadjuvant chemotherapy.

CHAPTER 2: LITERATURE REVIEWS

2.1 Epidemiology of Oral Squamous Cell Carcinoma

2.1.1 Incidence

Oral squamous cell carcinoma (OSCC) is defined as carcinoma with squamous differentiation arising from the mucosal epithelium. It constitutes more than 90% of oral cavity and oropharynx malignancies (El-Naggar et al., 2017).

Globally, lip and oral cancer ranked as the 16th most common cancer in the world, with over 350,000 new cases estimated in 2018 worldwide at a crude rate of 4.6 and age-standardized rate (ASR) of 4.0 per 100,000 persons. More than half (63.5%) of the cases occurred in Asia as 221,046 new cases were diagnosed from this region alone. Of these, the highest incidence of oral cancer was recorded from South-Central Asia countries (45.9% of all incidence cases) and India contributed highest incidence recorded by a country which was about 119,992 new cases (34.5 %) and highest ASR which was 9.1 per 100,000 persons a year (Figure 2.1). Oral cancer was also ranked as the second most common malignancy in India, Pakistan, Sri Lanka and Bangladesh (Warnakulasuriya, 2009a; Cheong et al.,2017; Shield et al., 2017; GLOBOCAN 2018).

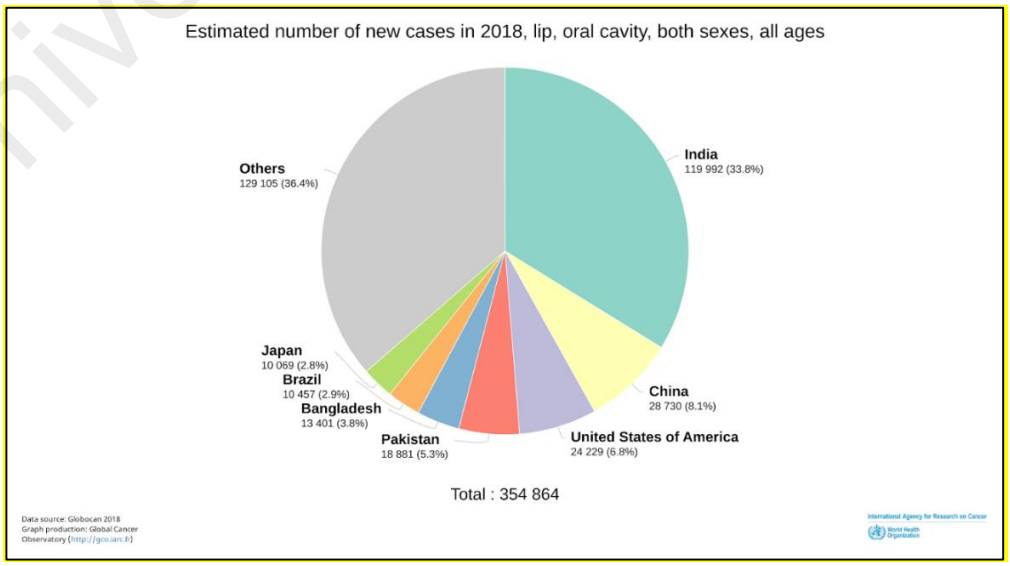

Figure 2.1 World estimated number of oral cancer new cases (Adapted from GLOBOCAN, 2018)

In South-East Asia, the incidence for new cases of oral cancer in 2018 were estimated to be about 16,818 cases (4.8%) while Malaysia was estimated to have 667 (0.2%) new cases of oral cancer which was the 18th most common cancer among the general population. It was also ranked as the 14th most common malignancies for both male and female in Malaysia and was estimated to have caused 327 mortality cases per year.

The ASR of mortality for oral cancer was about 2.0 per 100,000 persons globally, in which 177,384 deaths had been recorded. It increased significantly for South Central Asia regions and India (5.4 and 5.6 per 100,000 persons) with an estimated mortality of 98,851 and 72,616 persons respectively. In South-Eastern Asia countries and Malaysia, the ASR for mortality was much reduced at 1.3 and 1.1 per 100,000 persons respectively. Given that oral cancer was very much associated with lifestyle risk factors, the trends for incidence and mortality rates of oral cancer were projected to still be increasing for the coming years (Warnakulasuriya, 2009b; GLOBOCAN 2018).

2.2 Risk factors of oral cancer

The lifestyle behavioural risk factors for oral cancer are well known and have been reviewed by various authors. Among these, the most important risk factors were tobacco usage, betel quid chewing, and heavy alcohol drinking.

2.2.1 Tobacco

Tobacco consumption has been a global epidemic with Asia, Australia and the Far East by far the largest consumers, followed by America, Eastern Europe and Western Europe. Cigarette accounted for the largest share of manufactured tobacco products, with 96% of total value sales (Ranney et al, 2006). In a recent report by World Health Organization (WHO) on global tobacco epidemic in 2017, it was estimated that around 1.1 billion active smokers all over the world, in which 942 million was male smokers and the remaining 175 million female smokers. From these, the largest group of smokers came

from male smokers in middle income countries while around half of the world's female smokers lived in high income countries (WHO, 2017). In Malaysia, smoking prevalence among adults (>18 years old) was estimated to be 24.0 % in 2015, with majority of smokers being male (Clinical Practice Guidelines on Treatment of Tobacco Use Disorder, 2016).

The International Agency for Research on Cancer (IARC) classified tobacco smoking as a group 1 carcinogen for oral cavity (IARC 2012). About one fourth of oral cancer cases were caused by tobacco smoking. Its carcinogenicity has been causally linked to oral cancer and attributable mainly to tobacco-specific nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN), polycyclic aromatic hydrocarbons (PAH), such as benzo[a]pyrene and aromatic amines (IARC, 2004a). Tobacco smoking was associated with an increase between three to six folds risk of developing oral cancer compared to non-smokers (Gandini et al., 2008). It was also strongly proven that for smoking-associated risk, there was a dose-dependent relationship with both current daily tobacco consumption and lifetime exposure to tobacco smoke (IARC, 2004a; Thomas et al., 2007; Petti, 2009; Wyss et al., 2013).

2.2.2 Betel quid chewing

Betel quid generally consists of areca nut, part of the Piper betel plant (leaf or stem) and slaked lime (either as powder or paste). Some populations may have it together with tobacco and other spices, especially in regional areas of India and Southeast Asia. Considering that betel quid contains various carcinogens, and sites of betel quid chewing are commonly associated with higher rates of oral cancer, IARC has classified betel quid as human carcinogen group 1 (IARC, 2004b; Thomas et al., 2007). The carcinogenicity of betel quid in causing oral cancer has not been fully proven with strong evidence. Endogenous nitrosation has been demonstrated in chewer's mucosa, with consequent

production of potentially carcinogenic nitrosamines, such as 3-methylnitrosopropionitrile. Reactive oxygen species generation in the oral cavity due to auto-oxidation of polyphenols contained in areca nut and enhanced by the alkaline pH from slaked lime has also been reported (Nair et al., 2004; IARC, 2004b; Petti, 2009).

Guha et al. (2014) in their meta-analysis on betel quid chewing and the risk of oral and oropharyngeal cancers, showed that the risk of oral/oropharyngeal cancer increased with increasing daily amount and duration of chewing betel quid, in an exposure-dependent manner, independent of tobacco and alcohol use. Similar findings were also reported by Thomas et al. (2007) in their study on Papua New Guinea population, in which they showed that betel quid chewing was associated in dose-related manner with increased risk of oral cancer even when the betel quid does not contain tobacco.

2.2.3 Alcohol consumption

Alcohol is an established causal factor for some types of cancer and estimated to be responsible for 4.5% of the global burden of disease and injury (WHO, 2011). The mechanisms on how alcohol consumption caused its carcinogenic effect are copious but not fully understood. It was believed that acetaldehyde, which was ethanol's primary metabolite was responsible for part of the carcinogenicity of alcohol drinking on the liver and the upper aerodigestive tract, and can also cause direct DNA damage (Boffetta & Hashibe, 2006; Bagnardi et al., 2013). In a meta-analysis done by Bagnardi et al. (2015), it was reported that heavy alcohol consumption was associated with about 5-fold risk of getting oral and pharyngeal cancer, and was dose-dependent. It was also estimated that about 7-19% of oral cancers were attributable to heavy alcohol drinking (Petti, 2009).

Even though alcohol and tobacco are independent risk factors for oral cancer, it was proven that both can have a synergistic effect with a clear dose dependent in causing oral cancer (Boffetta & Hashibe, 2006; Maasland et al., 2014; Chinn & Myers, 2015). Alcohol

increases the permeability of oral mucosa, thus producing an alteration in morphology characterized by epithelial atrophy, which leads to easier penetration of carcinogens into the mucosa. As a result, the carcinogenic properties of both factors are likely to be enhanced in the presence of one another (Boffetta & Hashibe, 2006; McCullough & Farah, 2008; Maasland et al., 2014).

2.3 Treatment of oral squamous cell carcinoma

Management of patient with OSCC will usually involve multidisciplinary care. It includes specialists in surgery, radiation oncology, medical oncology, dental oncology, nursing, and speech pathology. The treatment of choice remains to be stage-dependent, according to several evidence-based clinical practice guidelines available worldwide (Scottish Intercollegiate Guidelines Network, 2006; Markopoulos, 2012; National Comprehensive Cancer Network, 2014; Chi et al., 2015).

For early stage of the disease, surgical approach is the principal single treatment modality used, with clear 1cm to 2cm margins (Omura, 2014; Chi et al., 2015; Chinn & Myers, 2015). In addition, neck dissection was commonly performed when lymph node disease was evident or when elevated risk of occult regional metastasis was suspected. For patients who presented with advanced stage of OSCC (Stage III or IV), multimodality treatment was recommended, with surgery remaining the first line treatment of choice, and options of combinations with adjuvant radiation or chemoradiation afterwards. The decision was often dictated by adverse findings from the surgery such as positive surgical margins, perineural or lymphovascular invasion, N2 or N3 lymph node disease, positive metastasis to lymph nodes level IV or V, and extracapsular extension of tumour in lymph nodes (Cooper et al., 2004; National Comprehensive Cancer Network, 2014; Wedemeyer et al., 2014).

There has been an increasing interest in treating OSCC with chemotherapy over the last 30 years. Three meta-analyses studies have concluded that chemotherapy was associated with a statistically significant advantage in survival of patients of OSCC, but the percentage was very low (4% absolute benefit at 2 & 5 years) (Munro, 1995; El-Sayed et al., 1996; Pignon et al., 2000). It was typically given as induction chemotherapy, also known as neoadjuvant chemotherapy (NAC), or concurrently given with radiotherapy after surgery and thus named chemoradiotherapy. The commonly used agents for chemotherapy were platinum-containing compounds (e.g: cisplatin, carboplatin), 5-fluorouracil and taxanes (e.g: paclitaxel, docetaxel).

2.4 Neoadjuvant chemotherapy

Neoadjuvant chemotherapy (NAC) (also called induction; preoperative or primary) referred to the application of systemic chemotherapy before a malignant tumour was treated with surgery or radiation therapy. The objective of this treatment was usually to reduce the tumour size, and subsequently to decrease the extent and invasiveness of surgery. The ultimate goal of NAC was to subsequently decrease local recurrence and decrease distant metastasis rate and since these factors affect survival, it has been hypothesized that survival may therefore improve as well (Kohno et al., 2000).

NAC, according to several studies, has potential benefits of improved overall survival, tumour reduction, local control, decreased recurrence, decreased distant metastases, organ preservation in resectable tumours, less need of postoperative radiotherapy and destructive surgical procedures. The adverse effects usually are myelosuppresion (thrombocytopenia, anaemia, leukopenia), nausea, vomiting, stomatitis, alopecia and facial edema (Pignon et al., 2000; Domenge et al., 2000; Kessler et al., 2007; Klug et al., 2009; Driemel et al., 2009; Patil et al., 2013 & 2014; Nanda & Mohiyuddin, 2015).

In spite of the reported benefits, contradicting results have emerged from other studies indicating that NAC has no added or significant benefits when compared to principal surgery treatment. Licitra et al. (2003) reported that the addition of NAC to standard surgery in patients with OSCC was unable to improve survival even though it might have a role in reducing the number of patients who needed to undergo mandibulectomy and/or adjuvant radiation therapy. The failure to improve survival was also reported by Zhong et al. (2013). However, patients with a clinical response or favourable pathologic response (<10% viable tumour cells) were reported as having superior overall survival and locoregional and distant control (Zhong et al., 2013).

The recent 8th edition of the American Joint Committee on Cancer (AJCC) cancer grading included a new staging category to the existing pTNM staging for patients who had neoadjuvant therapy with pathologic review of the tumour resection. The staging for these patients will be ypTNM instead of the usual pTNM staging. The reason for this change was due to the difference in the prognostic value of this treatment as compared to the primary surgical therapy (Rice et al., 2017).

2.4.1 NAC regimens

Older controlled studies have established cisplatin and continuous-infusion 5-fluorouracil (5-FU) as the standard NAC regimen for locally advanced squamous cell carcinoma of the head and neck patients (Paccagnella et al., 1994; Domenge et al., 2000; Pignon et al., 2000). However, despite inducing locoregional control, high response rates and a significant improvement in survival, cisplatin and 5-FU (PF) regimen in NAC was also associated with a relatively poor absolute rate of patient survival. In view to improve complete response rate to NAC, taxane agents had been introduced (Posner & Lefebvre, 2003). Combination of Doxetaxel and PF (TPF) were reported with significantly improved disease progression-free, overall survival, locoregional recurrence and distant metastasis in patients with head and neck squamous cell carcinoma in a few Phase III trial

and meta-analysis studies (Hitt et al., 2005; Vermoken et al., 2007; Posner et al., 2007; Lorch et al., 2011; Blanchard et al., 2013; Marta et al., 2015). Haddad et al. (2018) and Karabajakian et al. (2019) in their studies has also concluded that TPF was now the widely accepted gold standard for NAC.

In National Cancer Institute (IKN) where NAC in Malaysia was conducted, the regimen for OSCC patients was determined according to a systemic therapy protocol guideline published by the Ministry of Health in 2016. In this guideline, PF and TPF were the only regimens used for NAC in head and neck cancer patients (Table 2.1 and Table 2.2) for the moment. As Docetaxel was associated with cardiotoxic effects, TPF will usually be conducted in fit and young patients while PF was the first line drug of choice for older patients.

Table 2.1 Cisplatin-5FU regimen in IKN

	Cycle length (days) = 21		**Anti-emetic = 4**	
Drugs	**Dose (mg/m^2)**	**Route**	**Infusion time**	**Days**
Cisplatinum	75-100	IV	2 hours	1
5-Fluorouracil	750-1000	IV	2 hours	1-5

Table 2.2 TPF regimen in IKN

	Cycle length (days) = 21		**Anti-emetic = 4**	
Drugs	**Dose (mg/m^2)**	**Route**	**Infusion time**	**Days**
Docetaxel	60-75	IV	1 hour	1
Cisplatinum	75-100	IV	2 hours	1
5-Fluorouracil	750-1000	IV	24 hours	1-4

2.5 Tumour response to NAC

Tumour reduction or regression after NAC, has been shown to be highly correlated to disease free survival. The downstaging effects of NAC in which primary tumour volume reduction occurred, can later be usefully employed to lower the percentage of mandibulectomies and postoperative radiotherapy as required for advanced-stage tumours. Mandibulectomy is a kind of demolitive surgery which can cause significant functional impairment to the patient especially in masticatory and cosmetic aspects. Thus, NAC potentially provide relevant benefits in terms of organ- and functional- preservation (Licitra & Vermorken, 2004).

In addition, the shrinkage of the tumour, which normally occurred at the periphery of the tumour usually allowed a larger gap between the tumour edge and the critical structures, therefore may contribute to spare normal tissues from unnecessary radiation and potentially reduce final radiation dose (Licitra & Vermorken, 2004).

There were also a few studies that observed pathologic complete response (PCR) in patients of OSCC treated with NAC. PCR was defined as the lack of signs of cancer in tissue samples removed during surgery after treatment with radiation or chemotherapy (NCI Dictionary of Cancer Terms, 2018). Pathologic complete response was associated with improved loco-regional control and long term survival in these patients. Zhang et al. reported that PCR was obtained in about 25% of patients, and associated with 88.2% local control rate (Zhang et al., 2013).

Wedemeyer et al. analysed the association of tumour regression and further histopathological features (such as presence of ulceration, tumour necrosis, fibrosis, lymphangiosis, perineural invasion and others) after NAC with the overall survival (OS) of patients. They concluded that better tumour response to therapy was statistically significant associated with less locoregional recurrence. A complete tumour regression

was also associated with 88.9% 5-year OS, while the presence of ulceration, tumour necrosis and lymphangiosis showed a worse 5-year OS. They also suggested an adequate histopathological examination of neoadjuvantly treated cancer as necessary for a correct evaluation of tumour response (Wedemeyer et al., 2014).

2.6 Tumour regression models

The effects of tumour reduction or regression after NAC can be determined by histopathological assessment of the subsequent resection specimens. Tumour regression grading (TRG) is an attempt to stratify primary tumour response to the treatment, in terms of regressive changes which mostly refer to the amount of therapy induced fibrosis in relation to the residual tumour, or the estimated percentage of residual tumour in relation to the previous tumour site (Thies & Langer, 2013; Trakarnsanga et al., 2014). A review of relevant literature revealed a myriad of TRG systems available for squamous cell carcinomas and other malignancies. Below are several examples of the most commonly used TRG systems.

2.6.1 Braun-TRG system

Braun et al. (1989) examined 41 resected specimens histologically from patients who had advanced head and neck squamous cell carcinomas and treated with neoadjuvant radiochemotherapy. They came up with a 4-stage tumour regression grade, based on the regression scale by Salzer-Kuntschik et al. (1983) and Huvos et al. (1977) which were used for classification of chemotherapeutical pretreated osteosarcomas (Table 2.3). The proportions of vital, devitalized and organized tumour parts were marked on the paraffin slides; and the percentage of vital tumour parts of the whole tumour area were estimated.

Table 2.3 Braun Tumour Regression Model

Grade	Histologic hallmark
1	No vital tumour cells are detectable, instead a fibrosed and hyalinized connetive tissue with keratin pearls and giant cells is visible.
2	Fibrosed and scarred tissue with keratin pearls, giant cells and small clusters of vital tumour cells which do no exceed more than 5% of the whole lesion.
3	5%-50% of vital tumour cells in fibrosed scarred tissue.
4	More than 50% vital tumour cells, no signs of fibrosis and hyalinization.

These grades of regression were then evaluated as good (Grade 1,2) and bad responses (Grade 3,4) to the combined neoadjuvant radiochemotherapy. They also found that after a follow-up of 18 to 30 months, all patients that developed locoregional recurrence (14 patients; 34%) were bad responders to the neoadjuvant therapy, thus suggesting that histologic grade of tumour regression was a valuable prognosticator for response of the therapy.

2.6.2 Mandard-TRG system

Mandard et al. (1994) conducted a pilot study to investigate the features of regression of esophageal cancer after neoadjuvant chemoradiotherapy, in which the assessment was done quantitatively. Histologically, all the samples were grouped into 2 histologic groups; 1) no regressive changes of the tumour, and 2) regressive changes noted. They also listed the types of changes that needed to be assessed:

1. Cytologically, cancer cells showed cytoplasmic vacuolization and/or eosinophilia, nuclear pyknosis, and necrosis.

2. Stromal changes, including fibrosis, with or without inflammatory infiltrate, as well as giant cell granuloma around ghost cells and keratin. The fibrosis was supposed to be either dense with much collagen or edematous.

On the basis of these changes, Mandard TRG system was developed, and classified into 5 histologic grades (Table 2.4).

Table 2.4 Mandard Tumour Regression Grading Model

Grade	Description
1	Complete regression, with absence of histologically identifiable residual cancer cell and fibrosis extending through the different layers of the esophageal wall, with or without granuloma.
2	Presence of rare residual cancer cells scattered through the fibrosis.
3	An increase in the number of residual cancer cells, but fibrosis still predominated.
4	Residual cancer outgrowing fibrosis
5	Absence of regressive changes.

This TRG system was later widely applied on gastrointestinal cancers after neoadjuvant treatment, rendering it as one of the most widely used TRG system (Thies & Langer, 2013).

2.6.3 Dworak-TRG system

In 1997, Dworak and his associates developed a new tumour regression grading system when they investigated pathological features of rectal cancer after neoadjuvant radiochemotherapy. They examined surgical specimens of 17 patients presenting with clinically non-resectable rectal carcinoma, based on the tumour mass, fibrotic changes, irradiation vasculopathy, and peritumourous inflammatory reaction and graded the responses as follows (Table 2.5):

Table 2.5 Dworak Tumour Regression Grading Model

Grade	Description
0	No regression
1	Dominant tumour mass with obvious fibrosis and/or vasculopathy.
2	Dominantly fibrotic changes with few tumour cells or groups (easy to find).
3	Very few (difficult to find microscopically) tumour cells in fibrotic tissue with or without mucous substance.
4	No tumour cells, only fibrotic mass (total regression or response).

Dworak et al. found that all of these cases which had been classified as not curatively resectable before radiochemotherapy could be resected after the treatment. Even though they could not find total regression (Grade 4) in any of their cases, they suggested that neoadjuvant chemoradiotherapy can be a useful tool for tumour reduction and to increase operability.

2.6.4 Becker-TRG system

Becker et al. (2003) found that the histopathologic grading of regression in gastric carcinoma treated with NAC had significant correlation with survival. In their study, they developed a TRG model based on an estimation of the percentage of vital tumour tissue in relation to the macroscopically identifiable tumour bed that was evaluated histologically. The TRG model consists of three grades (Table 2.6).

Table 2.6 Becker Tumour Regression Grading Model

Grade	Description
1a	Complete regression (0% residual tumour)
1b	Subtotal tumour regression (<10% residual tumour per tumour bed)
2	Partial tumour regression (10-50% residual tumour per tumour bed)
3	Minimal or no tumour regression (>50% residual tumour per tumour bed)

Becker et al. also did not find any complete regression (Grade 1a) in any of the 36 specimens. In spite of that, they suggested that their patients benefited from the neoadjuvant treatment as their results showed a 5-year survival rate of 27% had been achieved.

2.6.5 Ryan-TRG system

The 5-point scale of Mandard's TRG model was modified by Ryan et al. in their prospective cohort study in 2005. They selected 60 patients with clinical and radiological evidence of locally advanced rectal cancer (T3/4 or N1/2), but without distant metastases and administered neoadjuvant chemoradiotherapy over a 5-week period. The modified 3-point grade was devised by combining TRG 1 and 2 to form one category, and combining TRG 4 and 5 into another category, giving rise to three distinct grades (Table 2.7).

Table 2.7 Ryan Tumour Regression Model/ Modified Mandard-TRG model

Mandard's TRG	Description	Three-point TRG
1	Complete regression, no viable cancer cells.	1
2	Presence of rare residual cancer cells scattered through the fibrosis.	
3	An increase in the number of residual cancer cells, but fibrosis still predominated.	2
4	Residual cancer outgrowing fibrosis	3
5	Absence of regressive changes.	

In the exercise of comparing 5-point TRG and 3-point TRG, it was found that full agreement was established by the pathologists for TRG 1 and 2, and disagreement was found in 16 specimens for TRG 3, 4 and 5 (Kappa statictic = 0.64) when using 5-point TRG. Meanwhile when using a 3-point score, majority of patients were reproducibly stratified to the correct categories (Kappa statistic = 0.84), with no disagreement about the complete responders, and disagreement only found in 6 specimens with partial or no response. Thus, they concluded that a 3-point grade yielded similar quality prognostic information, can be more easily implemented, and was more reproducible. They also suggested that even though not statistically significant, there was a trend towards a relationship between tumour response and cancer-specific survival.

2.6.6 Rodel-TRG system.

In a cohort study of patients with rectal carcinoma, treated by neoadjuvant chemoradiotherapy in 2005, Rodel et al. produced a new TRG system based on Dworak-TRG model (1997). The tumour regression of the primary tumour was semiquantitatively determined by the amount of viable tumour versus the amount of fibrosis (with

percentage- as compared to Dworak's TRG), ranging from no evidence of any treatment effect to a complete response with no viable tumour identified (Table 2.8).

Table 2.8 Rodel Tumour Regression Model

Grade	Description
0	No regression.
1	Minor regression (dominant tumour mass with obvious fibrosis in 25% or less of the tumour mass.
2	Moderate regression (dominant tumour mass with obvious fibrosis in 26%-50% of the tumour mass).
3	Good regression (dominant fibrosis outgrowing the tumour mass; i.e,more than 50% tumour regression)
4	Total regression (no viable tumour cells, only fibrotic mass).

From this study, Rodel et al. found that complete regression of the primary tumour (Grade 4) was associated with better control of disease in lymph nodes (ypN positive, 10%), intermediate tumour regression (Grade 2 + 3) had an intermediate risk of lymph node involvement (ypN positive, 32%) while poor tumour regression (Grade 0 + 1) was associated with adverse pathologic features such as higher incidence of nodal involvement (ypN positive, 42%) and more advanced ypT categories. These findings were in accordance with other TRG systems, which predicted that a better status of tumour regression was associated with better survival.

2.6.7 AJCC-TRG system.

In 2010, guidelines for grading rectal cancer response to neoadjuvant chemoradiotherapy was published by the AJCC and the College of American Pathology (CAP) and included in AJCC Cancer Staging Manual (7^{th} edition). This was meant for standardizing reporting and improving clinician access to information regarding the treatment response. This 4-category grading model was basically adapted from 3-

category grading scheme by Ryan and colleagues which differed on the complete absence of viable tumour cells (Grade 0) (Table 2.9).

Table 2.9 AJCC Tumour Regression Model

Grade	Description
0 (Complete response)	No viable tumour cells
1 (Moderate response)	Single cells or small groups of cancer cells
2 (Minimal response)	Residual cancer outgrown by fibrosis
3 (Poor response)	Minimal or no tumour kill; extensive residual cancer

The AJCC-TRG model was shown to be associated significantly with other known prognostic factors including angiolymphatic invasion, nodal metastasis, and pathologic stage (Mace et al., 2015). It was also considered as an independent prognostic factor for locally advanced rectal cancer, treated with neoadjuvant chemoradiotherapy (Zhang et al., 2016).

2.6.8 Comparison between tumour regression models

There were several studies done to evaluate and compare these regression models. Among the various TRG systems, only Braun-TRG and Mandard-TRG system have been used for head and neck cancer. In a study done by Wedemeyer and his colleagues in 2014, they compared these two TRG systems and made a conclusion that both produced comparable results in terms of tumour regression grades and suggested that any of these systems can be use alternatively (Wedemeyer et al., 2014). Mandard-TRG was preferred over Braun-TRG in this study as we observed that Mandard-TRG was used more

commonly in the assessment of other NAC-treated tumours and proven a good prognosticator.

When Ryan and colleagues tried to modify the Mandard-TRG system, they found that it was difficult to produce a high degree of agreement between the examiners with the 5-point score in Mandard-TRG. They attempted instead to improve reproducibility while still stratifying patients into prognostically significant groups using a modified 3-point grade and achieved satisfying results. They concluded that their 3-point grade yielded similar quality prognostic information, could be easily implemented and were more reproducible (Ryan et al., 2005). The Ryan-TRG system would later be adapted by the American Joint Committee on Cancer (AJCC) and College of American Pathologists (CAP) for the development of their own TRG system with the purpose of implementation nationally in the USA in 2010. Mace et al. (2015) in their attempt to validate the clinical relevance of the AJCC-TRG system found that known prognostic factors such as pathologic state, angiolymphatic invasion, histologic margin status, and tumour perforation were associated significantly with this TRG system. In addition, each grade was also associated significantly with distinct 5-year survival and recurrence probabilities. As AJCC-TRG was acknowledged as an independent predictor of oncologic outcome, Mace and his colleagues believed that AJCC-TRG system was superior to other previous regression grading systems.

Trakarnsanga et al. in his study in 2014 compared 6 different TRG system and found that the AJCC-TRG system was statistically significantly more accurate in predicting recurrence than the Mandard and Rodel-TRG systems ($p=0.002$ and 0.006, respectively). Apart from that, they also found that the five-tier system demonstrated no significant advantage over the three-tier TRG system. Since the AJCC-TRG system had a slightly higher concordance index compared to other TRG systems, they supported AJCC-TRG system to be adopted as the standard for classifying rectal cancer response to NAC.

However, in a recent study done to compare five TRG systems for gastric adenocarcinoma, Zhu et al (2017) found that even though all five TRG systems were significantly correlated with differentiation, postsurgical T category, postsurgical N category, tumour staging, lymphovascular invasion, perineural invasion, tumour size as well as statistically significant in univariate survival analysis, only Mandard-TRG system showed higher hazard ratio of death and disease progression for no/slightly response grade as compared to severe response grade. Thus, the authors recommended Mandard-TRG system in gastric carcinoma evaluation for prediction of survival.

After considering all aspects of the 7 TRG systems discussed above, we have short-listed 3 TRG systems deemed most suitable for use in our study. The selected TRGs are Mandard, Ryan and AJCC-TRG systems. Mandard-TRG system (1994) was chosen since there was evidence that it remains relevant in tumour regression grading in recent literature (Zhu et al., 2017). Ryan-TRG system was selected since 3-tier modification in the Ryan-TRG system was rated superior to the 5-tier Mandard-TRG system. And finally the AJCC-TRG system made the cut by being the most recent TRG system with generally favourable opinion. Among these three TRGs, Ryan-TRG and AJCC-TRG were never used for NAC treated OSCC patients in previous literature.

2.7 Biomarkers

At the moment, reliable methods to identify non-responding patients to NAC is only possible after surgery was done with consecutive histopathological assessment of the operative specimens. Therefore, it would be highly beneficial to explore new possibilities to predict tumour response to NAC, such as the use of biomarkers. (Wedemeyer et al., 2014)

2.7.1 Podoplanin

In a recent study by Kreppel et al. (2011), it was shown that podoplanin expression in patients with OSCC might serve as a prognostic factor to predict treatment response to NAC. (Kreppel et al., 2011).

2.7.1.1 Functional structure

Podoplanin (PDPN) is a 36- to 43-kDa mucin type transmembrane protein which has a wide variety of functions including regulation of organ development, cell motility and tumorigenesis and metastasis. It is necessary for proper development of heart, lungs, lymphoid organs and immune cells system. Apart from that, PDPN also plays pivotal functions in lymphangiogenesis, platelet production in the bone marrow and the immune response (Figure2.2) (Quintanilla et al., 2019). Even though PDPN expression patterns of the cells in the body have been well characterized, there was still little known about the physiological functions of this protein (Astarita et al., 2012; Quintanilla et al., 2019).

PDPN possesses the typical structure of a type I transmembrane mucin-like glycoprotein, with a heavily *O*-glycosylated ectodomain, a hydrophobic membrane spanning domain, and a short cytoplasmic tail of only nine amino acids. As the structure of PDPN lacks obvious enzymatic motifs, hence it must exert its cellular functions through protein-protein interactions (Figure 2.3).

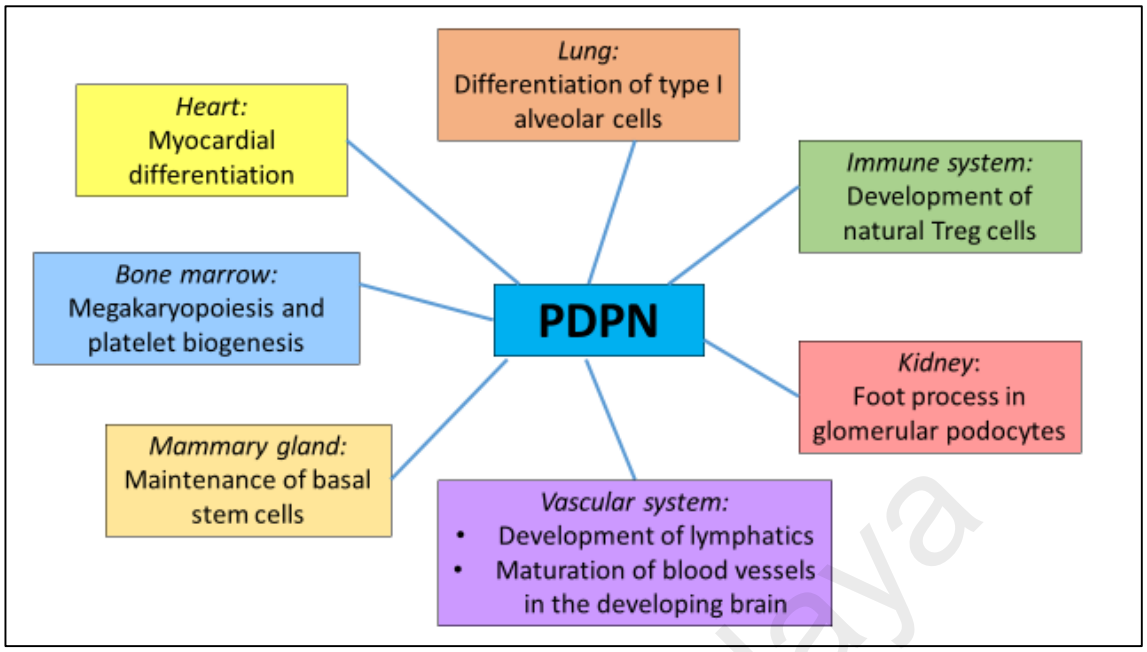

Figure 2.2 Summary of the involvement of podoplanin in organogenesis and differentiation.

(Taken from Quintanilla et al., 2019)

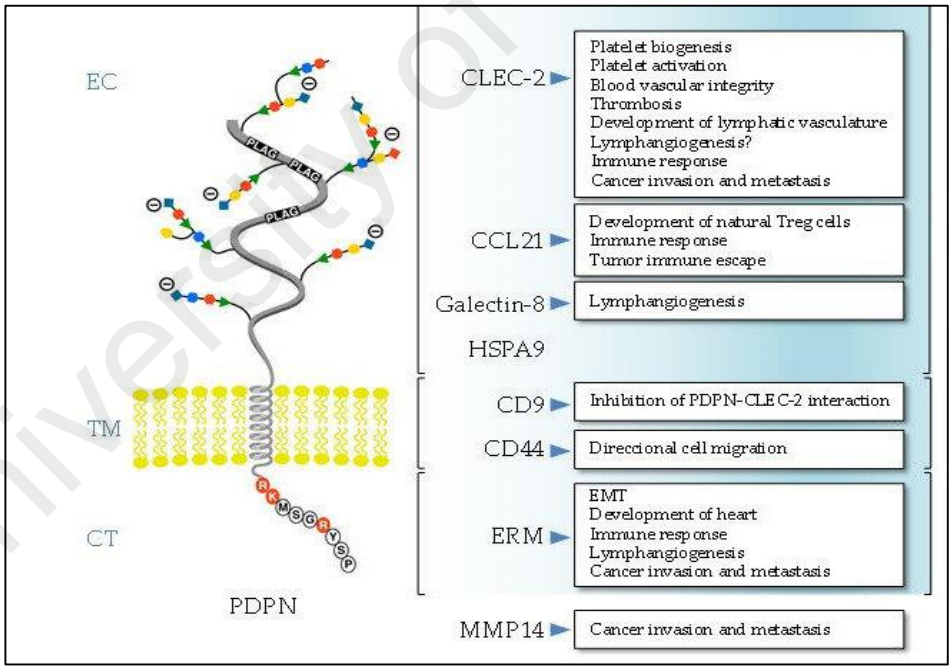

Figure 2.3 Schematic representation of PDPN structure.

(Taken from Quintanilla et al., 2019)

2.7.1.2 Distribution

PDPN was specifically expressed in lymphatic endothelial cells, but not in blood endothelial cells (Kahn & Marks, 2002). In addition, it was also widely expressed in various tissues and cell types, such as glomerular podocytes (hence its name), type I alveolar cells, osteocytes, mesothelial cells, choroid plexus, glia cells, some type of neurons, and different types of fibroblasts (Quintanilla et al., 2019).

2.7.1.3 Podoplanin and OSCC

As it was a specific marker for lymphatic vessels, and an increase in lymphangiogenesis was often associated with metastasis and poor prognosis in cancer patients, the numbers of PDPN positive vessels in a tumour was often used as a diagnostic marker (Swartz & Lund, 2012). In studies done by Yuan et al. (2006) and Huber et al. (2011), patients with high levels of PDPN expression in tumour cells had a higher frequency of lymph node metastasis than the patients with low level of PDPN. It was also found that high level of PDPN expression was associated with decreased patient overall survival, particularly disease-specific survival, in oral cancers (Yuan et al., 2006). Kreppel et al. (2010) had also made similar findings, and suggested that PDPN might play a role in lymphatic spread and in tumour invasion and progression.

Even though PDPN is known for its prognostic value in OSCC patients treated with primary surgery followed by radiochemotherapy, its potential predictive value with regards to neoadjuvant therapy has only been investigated in one study conducted by Kreppel et al. in 2011. They found that high expression of PDPN in pretreatment biopsy specimens was significantly associated with non-regression of the tumour and poor OS (Kreppel et al., 2011).

2.7.2 VEGF

Angiogenesis is the growth of new microvessels, and it depends on the motility, proliferation and tube formation of endothelial cells. In a review done by Cosway et al. (2015), they suggested that tumour angiogenesis was also a predictive marker for response to induction chemotherapy, as low density of microvessels were associated with partial and complete response to this treatment.

2.7.2.1 Functional features

The vascular endothelial growth factor (VEGF) is a 46 kDa heparin-binding homodimeric glycoprotein, with a highly conserved receptor-binding cystine-knot structure similar to the platelet-derived growth factors (Figure2.4) (Muller et al., 1997; Holmes & Zachary, 2005). Table 2.10 shows types of VEGF family members (Muller et al).

Table 2.10 VEGF Family

Type of VEGF	Function
VEGF-A	Angiogenesis
VEGF-B	Embryonic angiogenesis (myocardial tissue)
VEGF-C	Lymphangiogenesis
VEGF-D	Development of lymphatic vasculature surrounding lung bronchioles
PlGF	Vasculogenesis, also needed for angiogenesis during ischemia, inflammation, wound healing and cancer.

VEGFs act through a family of cognate receptor kinases in endothelial cells in order to stimulate blood-vessel formation (angiogenesis), and among the VEGFs, VEGF-A (also well-known as VEGF) possesses important roles in vascular development as well as in diseases involving abnormal growth of blood vessels (Holmes & Zachary, 2005).

VEGF was known to be a highly potent and leading angiogenic protein factor that induces proliferation, differentiation and migration of vascular endothelial cells, and promotes endothelial cells survival by preventing apoptosis (Gupta et al., 1999; Kyzas et al., 2005; Kim et al., 2015).

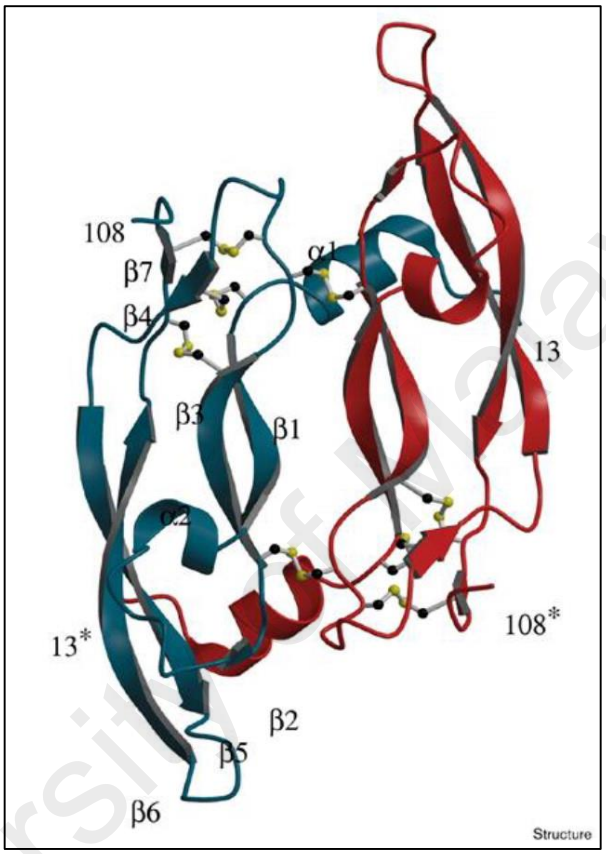

Figure 2.4 VEGF structure

(Taken from Muller et al., 1997).

2.7.2.2 Distribution

VEGF was believed to be expressed as early as embryonic day 7 in embryonic endoderm, and later expressed in the mesenchyme and neuroectoderm of the head. The expression declined in most tissues in the weeks after birth and was relatively low in most adult organs, excepts in few vascular beds, lung alveoli, kidney glomeruli and heart. The expression has been found to be upregulated during specific physiological processes such as development of the endocrine corpus luteum in pregnancy, wound healing and tissue

repair, and in diseases associated with neovascularization (formation of new blood-vessels). VEGF is produced by various cell types, including aortic vascular smooth muscle cells, keratinocytes, macrophages and many other tumour cells (Dvorak et al., 1995).

2.7.2.3 VEGF and OSCC

For oral cancer, many studies concluded that VEGF expression was a prognostic factor in patients with OSCC, and it correlated with poor prognosis for these patients (Kim et al., 2015; Uehara et al., 2004; Smith et al., 2000; Maeda et al., 1998)

Zhao et al. (2013) in his meta-analysis study suggested that overexpression of VEGF was a prognostic factor for patients with head and neck cancers, and the prognostic effects might be influenced by other related factors such as clinical stage, differentiation or lymph node metastasis. He also found that there was a significant positive association between VEGF overexpression and lymph node metastasis. This was similar to the findings by Cheng et al. (2011) who also correlated VEGF overexpression to advanced clinical stages, poorer cumulative survival as well as worse prognosis.

CHAPTER 3: METHODOLOGY

3.1 Study design

This was a retrospective study involving patients diagnosed with oral squamous cell carcinoma who underwent neoadjuvant chemotherapy prior to surgical removal of the tumour in Malaysia (Hospital Tengku Ampuan Rahimah, Klang & Hospital Shah Alam). The ethical approval of this study was obtained from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [Ethical Approval Code: DF OS 1717/0034 (P)]. This research was supported by the BKP grant (BK051-2017).

3.2 Materials

3.2.1 Tissue samples

Initially we managed to identify 38 patients diagnosed with OSCC between 2012 and 2017, planned for NAC prior to surgical removal of the tumour. However, a number of these patients did not last through the NAC or proceeded until the surgical stage due to developing severe reaction or the treatment were intended as palliative only. Finally, a total of 14 cases were eventually selected based upon the inclusion and exclusion criteria and the availability of the specimens. Formalin-fixed paraffin-embedded (FFPE) blocks of biopsy and surgical specimens from these patients that were routinely processed for histopathological diagnoses, were obtained from the archives of Oral Pathology Diagnostic Laboratory and Malaysian Oral Cancer Database & Tissue Bank System (MOCDTBS), coordinated by Oral Cancer Research and Coordinating Centre (OCRCC), Faculty of Dentistry, University of Malaya. In addition, unstained silanized slides for 4 biopsy specimens which were diagnosed in other centers were also obtained from Hospital Raja Perempuan Bainun (2), Hospital Sultan Ismail (1) and Institute of Medical Research (1) respectively.

3.2.1.1 Inclusion criteria

1. Samples that are diagnosed histologically as OSCC, treated with NAC and later underwent surgical removal of the tumour (complete biopsy and surgical specimens).
2. Patients with complete information (medical record, treatment,etc)

3.2.1.2 Exclusion criteria

1. OSCC samples from patients who do not have adequate clinical and follow-up data.

3.2.1.3 Patient's data/information

Patients' records and database from the Department of Oral Maxillofacial Surgery, HTAR were referred to for the following information:

1. Patients' characteristics and demographic profiles (age, gender)
2. Clinicopathologic parameters or disease (tumour) profile: stage/ grade of cancer, histological type, cancer site from which the samples were taken from, specimen characteristics.
3. Disease management: diagnosis, treatments (regimens used, number of cycles, date of surgery, follow up, survival status, etc).

3.2.2 Antibodies

Monoclonal antibodies of Podoplanin (Dako; Product Code M3619; Batch No. 10136947) and VEGF (VEGF-A) (Dako; Product Code M7273; Batch No 20050510) were purchased from Bita Lifesciences Sdn Bhd.

The primary antibodies and the methods used in the staining procedures were performed with modifications according to the manufacturer's guidelines as below:

Table 3.1 Brief details of the antibody and IHC protocol

Primary Antibody	Podoplanin	VEGF
Manufacturer	Dako	Dako
Control Tissue	Human (Lymphangioma tissue)	Human tonsil
Dilution	1:200	1:25
Antigen retrieval buffer & pH	Citrate buffer (pH6.0)	Tris-EDTA buffer (pH 9.0)
Incubation period	30min	Overnight
Wash buffer & pH	PBS (pH7.4)	PBS (pH7.4)

3.3 Methods

The flow of this study is illustrated in Figure 3.1:

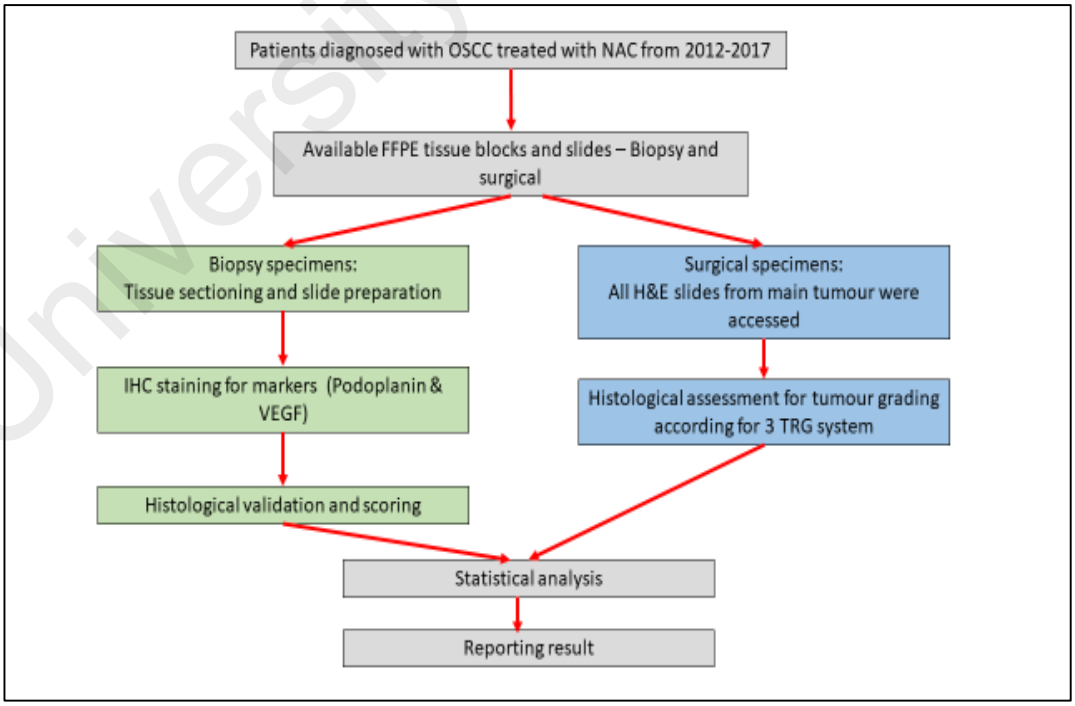

Figure 3.1 Flow of methodology

3.3.1 Specimens processing

All biopsy tissue specimens had been fixed in 10% buffered formalin prior to processing and embedding in paraffin blocks. Five sections from each tissue block were then sliced in 5 µm thickness and mounted on sialinized slides. Sections for Haematoxylin & Eosin staining were incubated at 60ºC for one-hour prior to staining. Sections for immunohistochemistry staining were also incubated at 60º for one hour for deparaffinization.

3.3.2 Tissue staining

3.3.2.1 Haematoxylin and Eosin (H&E) staining

One section of 5 µm thickness from biopsy specimens of each case were stained with H&E and then assessed for suitability of immunohistochemical staining by confirming the presence of adequate tissue representing the tumour. The H&E staining method used are described in Appendix A.

3.3.2.2 Immunohistochemical (IHC) staining

IHC staining were done on all 14 biopsy samples, 3 positive control tissue slides for each antibodies. Similar immunohistochemical procedures, wash buffer and temperature were utilized for both the antibodies. However, the antigen retrieval buffer, pH and the incubation period differed from each other. IHC staining was performed using the Dakocytomation REAL EnVision Detection System-HRP according to the manufacturer's specifications. The procedures followed for immunohistochemical studies are described in Appendix B.

3.4 Histopathological examination for tumour regression

For all 14 samples, H&E stained slides of the surgical specimens were re-evaluated and regression grading was performed according to three tumour regression models (Mandard et al., 1994; Ryan et al., 2005; Edge et al., 2010). The grading was done by 2

assessors (one oral pathologist and one oral pathologist trainee). The assessors were blinded to the patient outcome and in cases where grading was unclear, the case was reviewed together and grading was determined by consensus. Calibration exercise between the two assessors was performed to evaluate the inter-observer agreement (refer to section 4.3.1 or Appendix C-1). The grades are defined as follows:

Table 3.2 Criteria of three tumour regression systems.

TRG system	Grade	Description
Mandard-TRG	1	Complete regression. No residual cancer cells.
	2	Rare residual cancer cells.
	3	Fibrosis outgrowing residual cancer cells.
	4	Residual cancer outgrowing fibrosis.
	5	Absence of regressive changes.
Ryan-TRG	1	Complete regression/ rare residual cancer cells.
	2	Moderate regression. Fibrosis outgrowing residual cancer cells.
	3	Minimal/ absence of regression.
AJCC-TRG	0	Complete response. No viable tumour cells.
	1	Moderate response. Single cells or small groups of cancer cells.
	2	Minimal response. Residual cancer outgrown fibrosis.
	3	Poor response. Minimal or no tumour kill; extensive residual cancer.

3.5 Analysis of immunostaining

3.5.1 Calibration

Similar to the histopathological tumour regression grading, the IHC scoring of all the stained slides was performed by two independent assessors who were blinded to the clinico-pathological details pertaining to the patients. Any discrepancy in scoring between the two assessors was re-evaluated and a consensus score was determined. Inter-observer level of agreement was assessed by Intraclass Correlation Coefficient on SPSS software version 23 (refer to 4.4.1 & 4.4.2 or Appendix C).

3.5.2 Scoring

The IHC staining for both podoplanin and VEGF were analyzed semiquantitatively using Immunoreactive Score (IRS). As both markers were expressed in the cytoplasm and membrane of the tumour cells, five high-power fields were selected randomly from the slides. The percentage of immunopositive cells was quantified as follows; 0 = negative; 1 = 1% - 10%; 2 = 11% - 50%; 3 = 51% - 80%; 4 = >80% of positive cells. The intensity score was quantified using the following scores; 0 = negative; 1 = weak; 2 = moderate; 3 = strong. The IRS score was then produced by multiplying the percentage and intensity scores of the stained cells which will range from 0 to 12 (Table 3.3). The mean score of all 5 fields were then calculated and recorded for each of the cases. The final score was the mean score of the two independent examiners.

Table 3.3 Final IRS score for podoplanin and VEGF

Percentage **Intensity**	**0=0**	**1=1-10%**	**2=11-50%**	**3=51-80%**	**4=>80%**
0 = Negative	0	0	0	0	0
1 = Weak	0	1	2	3	4
2 = Moderate	0	2	4	6	8
3 = Strong	0	3	6	9	12

3.6 Statistical analysis

Statistical analysis was carried out using SPSS software (version 23). The association between the tumour regression grading with the socio-demographic and clinico-pathological parameters of the samples was analyzed by Fisher exact tests. The expression of podoplanin and VEGF were also analyzed against tumour regression grading with the use of Fisher exact tests. A *p*-value <0.05 is statistically significant.

CHAPTER 4: RESULTS

4.1 Socio-demographic findings

Tissue specimens from biopsy and surgical procedure were obtained from patients diagnosed with OSCC from 2011 to 2017. The samples were almost equally distributed among males and females aged between 38-70 years old with a mean age of 58.14. Majority of patients were aged above 45 years (85.7%). The rest of the socio-demographic findings are included in Table 4.1, and Figures 4.1 and 4.2.

Table 4.1 Socio-demographic characteristics

Variables	Value (n=14)	%
1. Age		
Mean ±standard deviation, range	58.14 ±9.99, 38-70	
< 45 years old	2	14.3
> 45 years old	12	85.7
2. Gender		
Male	8	57.1
Female	6	42.9
3. Race		
Malay	4	28.6
Chinese	2	14.3
Indian	8	57.1

4.2 Clinicopathological findings

A total of 14 OSCC cases that underwent neoadjuvant chemotherapy before surgical resection were selected, comprising 8 from buccal mucosa, 3 from tongue and 3 from other subsites of oral cavity (floor of mouth and alveolus). According to histopathological assessment, half of the cases were moderately differentiated SCC (50%). Most of the cases (71.4%) had tumour metastasis to the lymph node (LN) and from these, 3 of them

exhibited extracapsular spread (ECS) of the tumour from the LN. Apart from that, all the tumours examined in this study exhibited non-cohesive pattern of invasion (Type III & IV). The rest of the clinicopathological parameters are tabulated in Table 4.2.

Table 4.2 Clinico-pathological characteristics

Characteristics	Value (n=14)	%
1. Primary Tumour		
Buccal mucosa	8	57.1
Tongue	3	21.4
Floor of mouth	2	14.3
Alveolus	1	7.1
2. Tumour size		
T1-T2	4	28.6
T3-T4	10	71.4
Total	14	100.0
3. pTNM staging		
Stage I & II	2	14.3
Stage III & IV	12	85.7
Total	14	100.0
4. Surgical margin status		
Involved	4	28.6
Not involved	10	71.4
Total	14	100.0
5. Lymph node metastasis (ypN)		
N0	4	28.6
N1, N2, N3	10	71.4
Total	14	100.0

6. Extracapsular spread		
Yes	3	30.0
No	7	70.0
Total	10	100.0
7. Differentiation		
Well	5	35.7
Moderate	7	50.0
Poor	2	14.3
Total	14	100.0
8. Pattern of invasion		
Cohesive	0	0.0
Non-cohesive	14	100.0
Total	14	100.0
9. Perineural invasion		
Yes	4	28.6
No	10	71.4
Total	14	100.0
10. Lymphovascular invasion		
Yes	0	0.0
No	14	100.0
Total	14	100.0

4.3 Tumour regression grading

4.3.1 Patient distribution according to TRG

There were 2 cases with complete regression/response which were graded as 1 for Mandard-TRG and 0 for AJCC-TRG. For moderate regression (grade 3 for Mandard; grade 2 for Ryan & AJCC), there was only 1 case seen. Meanwhile 9 cases were graded

as having poor regression (Grade 3 for Ryan & AJCC) and 4 cases as having absence of regression (Grade 5 for Mandard). Interobserver measure of agreement for these grading was good (Kappa = 0.62 for Mandard TRG; Kappa = 0.74 for AJCC) and very good (Kappa = 1.00) for Ryan TRG (Table 4.3). Examples of the histological appearances in the TRG are shown in Figure 4.1 to Figure 4.5.

Table 4.3 Patient distribution according to three different TRGs

TRG system	Distribution, n (%)		
	Mandard TRG	**Ryan TRG**	**AJCC TRG**
Grade 0	-	-	2 (14.3)
Grade 1	2 (14.3)	4 (28.6)	2 (14.3)
Grade 2	2 (14.3)	1 (7.1)	1 (7.1)
Grade 3	1 (7.1)	9 (64.3)	9 (64.3)
Grade 4	5 (35.7)	-	-
Grade 5	4 (28.6)	-	-

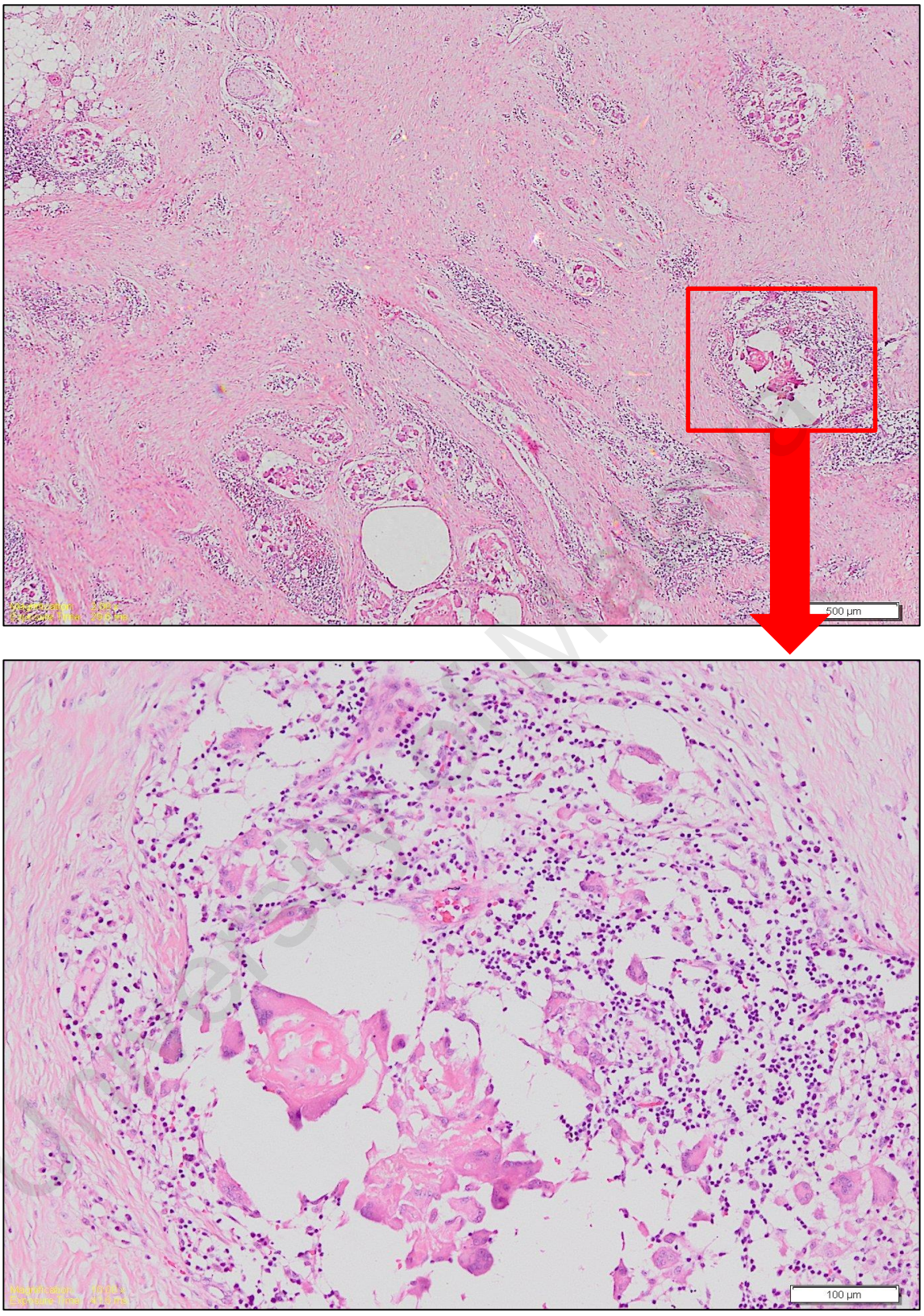

Figure 4.1 Complete regression
(A) Low power magnification (20x): Grade 1 Mandard TRG& Ryan TRG; Grade 0 AJCC TRG. (B) Higher power magnification (100x): Tumour cells degeneration with presence of multinucleated giant cells.

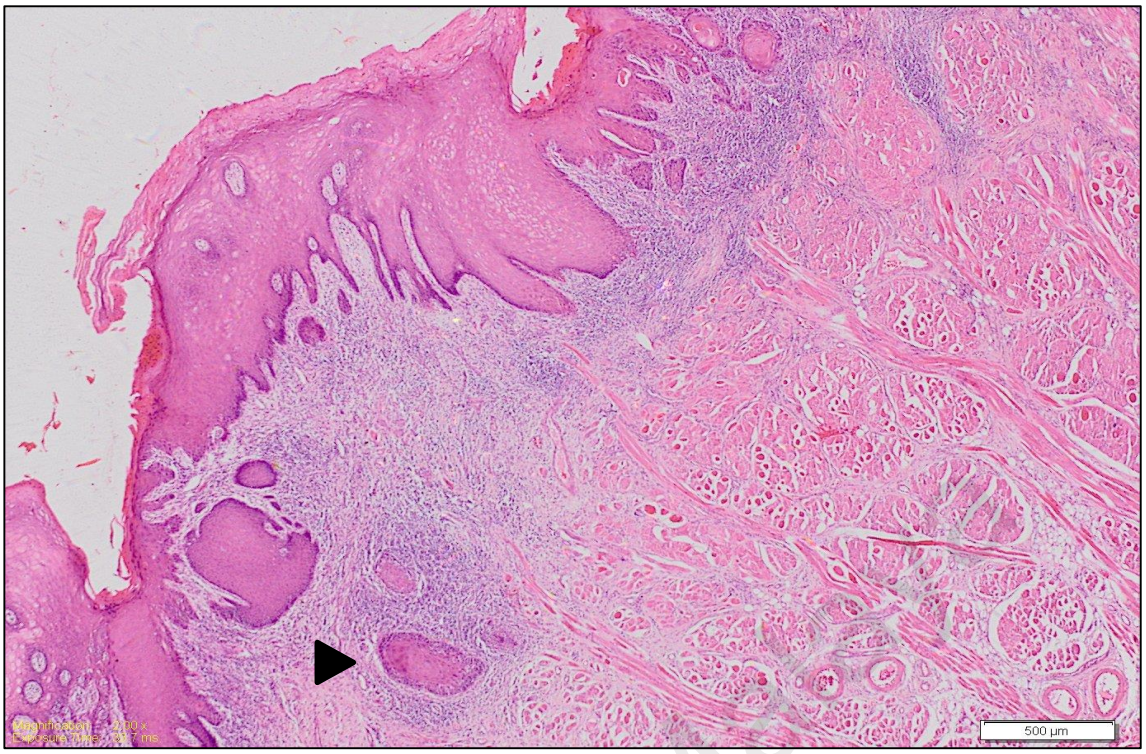

Figure 4.2 Moderate regression.
Presence of rare viable tumour cells (black arrow). Grade 2 for Mandard TRG; Grade 1 for Ryan TRG & AJCC TRG. [20x magnification].

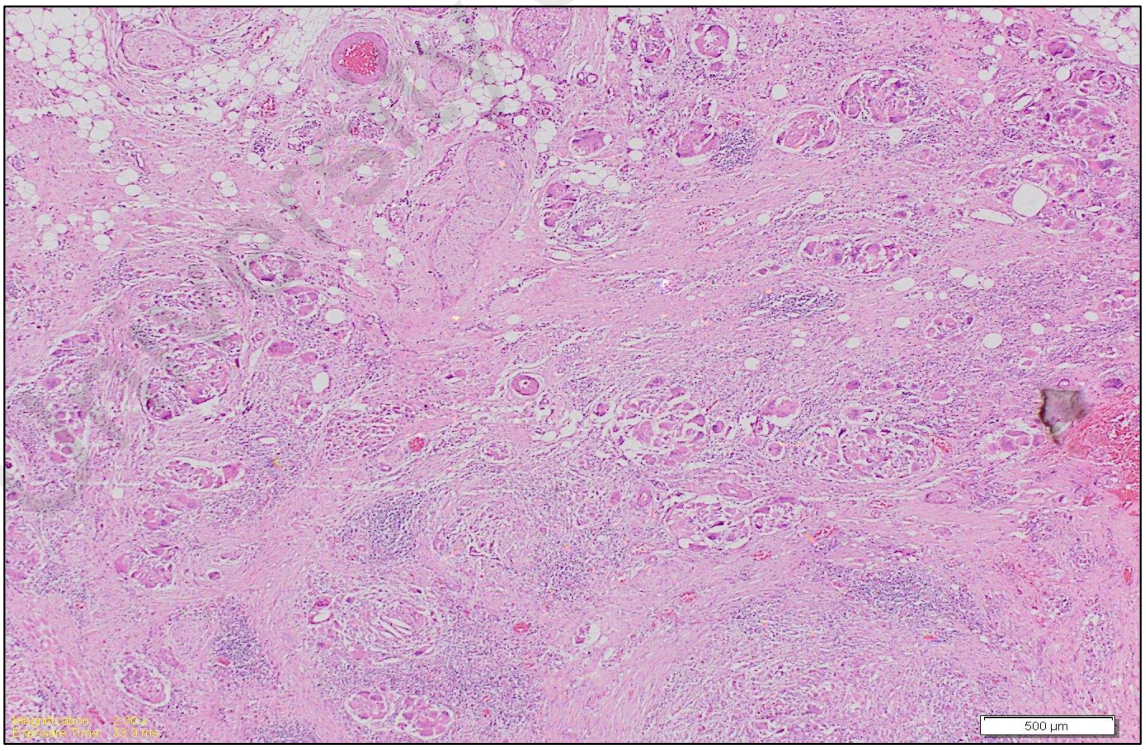

Figure 4.3 Mild tumour regression.
Increase in number of tumour cells, but fibrosis and tumour response to neoadjuvant chemotherapy predominated. Grade 3 for Mandard TRG; Grade 2 for Ryan & AJCC TRG. [20x magnification].

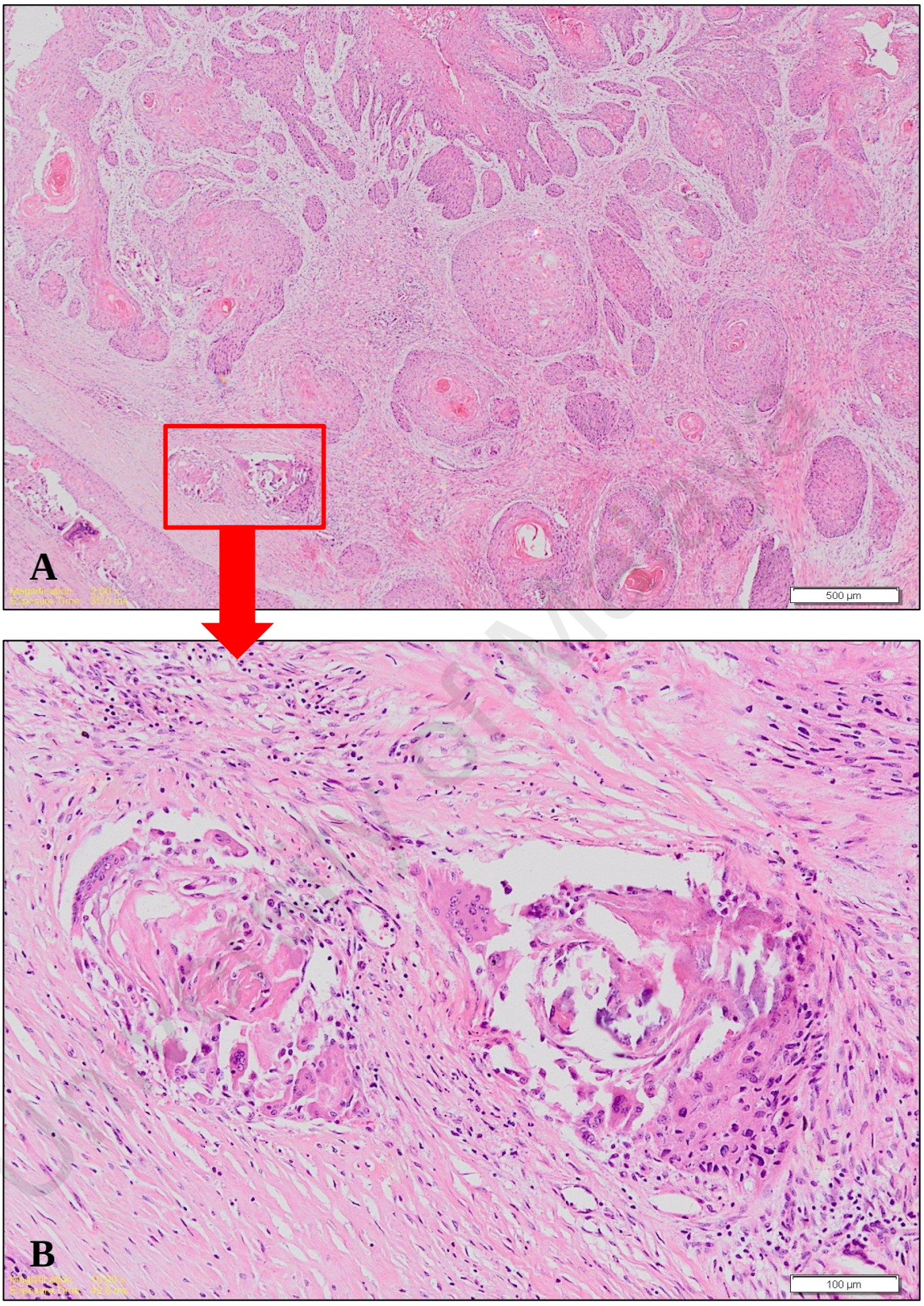

Figure 4.4 Poor response to NAC.
(A) Tumour cells outgrowing fibrosis [20x magnification]. (B) Tumour response to NAC with presence of fibrosis and multinucleated giant cells [100x magnification]. Grade 4 Mandard TRG; Grade 3 Ryan & AJCC TRG.

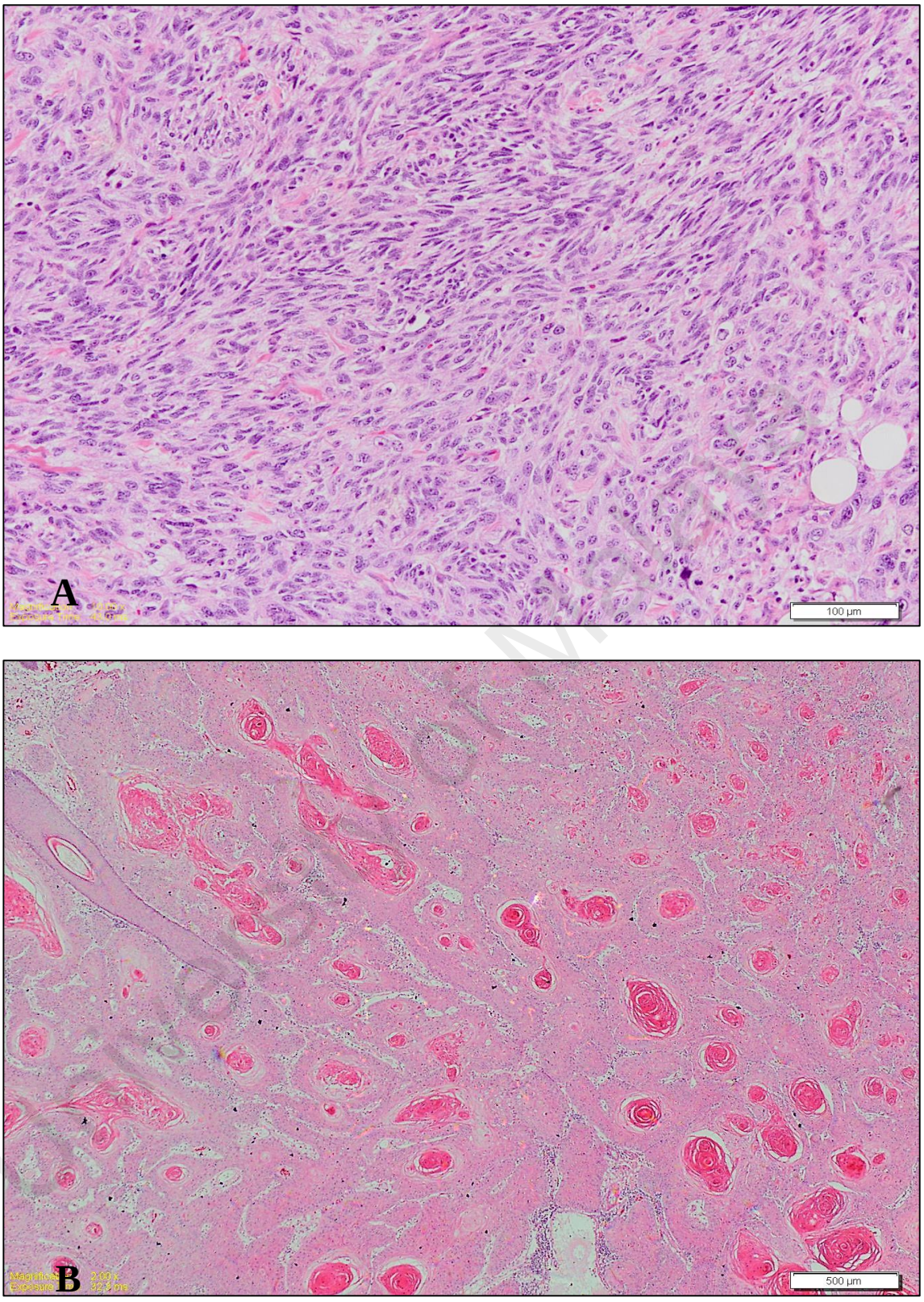

Figure 4.5 Absence of tumour regression.
Grade 5 for Mandard TRG; Grade 3 for Ryan and AJCC TRG. (A) Poorly differentiated OSCC [100x magnification]. (B). Well differentiated OSCC. [20x magnification].

4.3.2 Association between TRG and clinicopathological parameters

Association between AJCC-TRG model with clinicopathological parameters are shown in Table 4.4. Tumour regression graded with AJCC-TRG model showed a significant correlation with tumour size (p=0.001). Majority of samples (90%) with T3 and T4 tumour size, showed little regression and were graded as Grade 3 according to AJCC-TRG model. No other significant results were observed with other parameters. However, among 12 samples with Stage III and IV tumours, majority (75%) were graded as Grade 3 (mild or absence of regression). In addition, majority (80%) of tumours that metastasized to lymph nodes (n=10) were also graded as having mild or absence of regression (Grade 3).

Table 4.4 AJCC TRG association with clinico-pathological parameters

Characteristics	Value n (%)	AJCC-TRG, n (%)				*p**
		0	1	2	3	
1. Primary Tumour						
Buccal mucosa	8 (57.1)	2 (25.0)	0 (0.0)	1 (12.5)	5 (62.5)	
Tongue	3 (21.4)	0 (0.0)	1 (33.3)	0 (0.0)	2 (66.7)	0.710
Floor of mouth	2 (14.3)	0 (0.0)	1(50.0)	0 (0.0)	1 (50.0)	
Alveolus	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)	1(100.0)	
Total	14(100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
2. ypTumour size						
T1-T2	4 (28.6)	2(50.0)	2 (50.0)	0 (0.0)	0 (0.0)	
T3-T4	10 (71.4)	0 (0.0)	0 (0.0)	1(10.0)	9 (90.0)	**0.001**
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
3. pTNM staging						
Stage I & II	2 (14.3)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	
Stage III & IV	12 (85.7)	1 (8.3)	1 (8.3)	1 (8.3)	9 (75.0)	0.110
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
4. Surgical margin status						
Involved	4 (28.6)	0 (0.0)	0 (0.0)	1(25.0)	3 (75.0)	
Not involved	10 (71.4)	2 (20.0)	2 (20.0)	0 (0.0)	6 (60.0)	0.395
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	

5. Lymph node metastasis (ypN)						
N0	4 (28.6)	1 (25.0)	1 (25.0)	1(25.0)	1 (25.0)	
N1, N2, N3	10 (71.4)	1 (10.0)	1 (10.0)	0 (0.0)	8 (80.0)	0.167
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
6. Extracapsular spread						
Yes	3 (30.0)	0 (0.0)	1 (33.3)	0 (0.0)	2 (66.7)	
No	7 (70.0)	1 (14.3)	0 (0.0)	0 (0.0)	6 (85.7)	0.533
Total	10 (100.0)	1 (10.0)	1 (10.0)	0 (0.0)	8 (80.0)	
7. Differentiation						
Well	5 (35.7)	1 (20.0)	1 (20.0)	0 (0.0)	3 (60.0)	
Moderate	7 (50.0)	1 (14.3)	1 (14.3)	1(14.3)	4 (57.1)	1.000
Poor	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	2(100.0)	
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
8. Pattern of invasion						
Cohesive	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Non-cohesive	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	NA**
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
9.Perineural invasion						
Yes	4 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)	4(100.0)	
No	10 (71.4)	2 (20.0)	2 (20.0)	1(10.0)	5 (50.0)	0.520
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
10. Lymphovascular invasion						
Yes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
No	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	NA**
Total	14 (100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	

* : Test perfomed: Fisher's Exact test; Level of significance: $p<0.05$

**: Statistic test not applicable.

4.4 Expression of markers

4.4.1 Podoplanin expression

Podoplanin expression was seen in the cellular membrane as well as within the cytoplasm. In our samples, apart from being positively expressed in the endothelial cells lining the lymphatic channels, which served as our internal control, podoplanin was also expressed in some of the basal cell layer of the hyperplastic and dysplastic surface epithelium. In tumour cells, two distinct patterns of expression were displayed; diffuse expression in most tumour cells (Figure 4.6 (D)) and focal expression at the proliferating periphery of the tumour cells nests with no expression in the central areas (Figure 4.6 (C)). In the second pattern, the central areas of the tumour nests or islands usually contained more differentiated cells, thus this pattern mimicked the pattern seen in the dysplastic surface epithelium. Interobserver measure of agreement for podoplanin scoring was good (Kappa= 0.685). Majority of our samples (71.4%) showed moderate staining of podoplanin while only 1 sample (7.1%) was scored as having negative or very weak staining (Table 4.5). Examples of different scoring for PDPN staining are shown in Figure 4.6.

Table 4.5 Distribution of podoplanin expression

Staining	Value, n (%)
Negative	1 (7.1)
Weak	1 (7.1)
Moderate	10 (71.4)
Strong	2 (14.3)

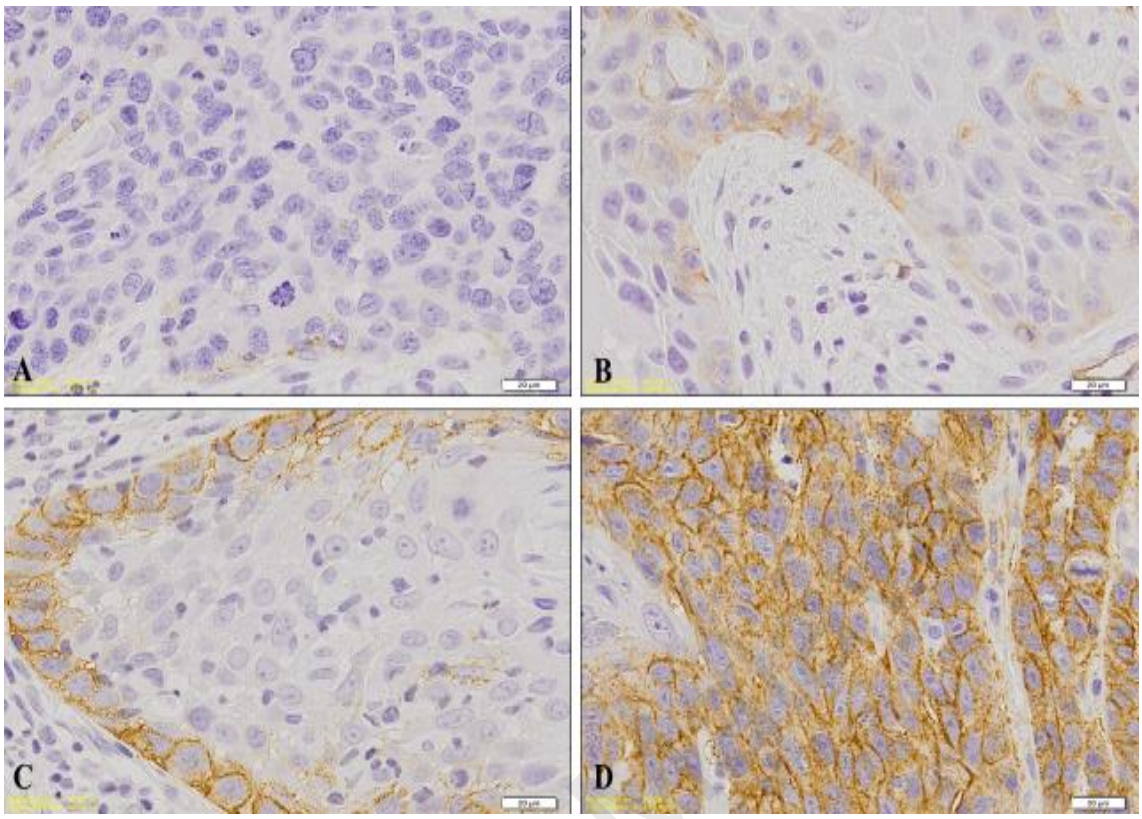

Figure 4.6 Podoplanin expression scoring.
(A) Negative staining in OSCC. (B) Mild positive staining in OSCC. (C) Moderate positive staining in OSCC. (D) Strong positive staining in OSCC.

4.4.2 VEGF expression

In all our samples, VEGF was expressed in cytoplasmic and membrane of the tumour cells. Similar to podoplanin expression, VEGF also showed two distinct patterns of expression, which were diffuse cytoplasmic expression (Figure 4.7) and stronger expression in the peripheral edge of the tumoural islands (Figure 4.8). There was no sample scored as having strong expression of VEGF while more than half of the samples (57.1%) had moderate expression of VEGF. Interobserver measure of agreement for these grading was good (Kappa=0.731). Table 4.6 shows the distribution of VEGF expression in our study. Examples of different scoring for VEGF staining are shown in Figure 4.9.

Table 4.6 Distribution of VEGF staining

Staining	Value, n (%)
Negative	2 (14.3)
Weak	4 (28.6)
Moderate	8 (57.1)
Strong	0 (0.0)

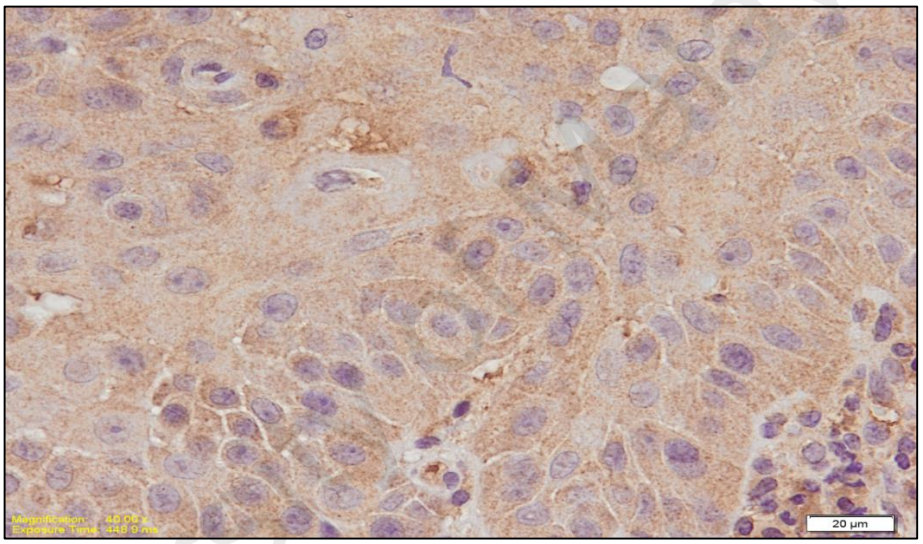

Figure 4.7 Diffuse VEGF expression.

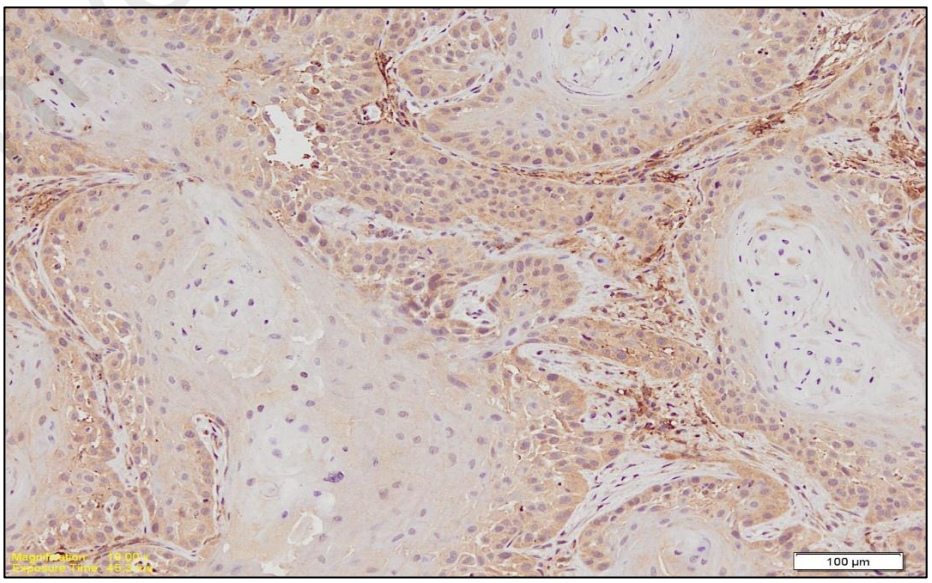

Figure 4.8 Stronger VEGF expression in peripheral edge of the tumour islands.

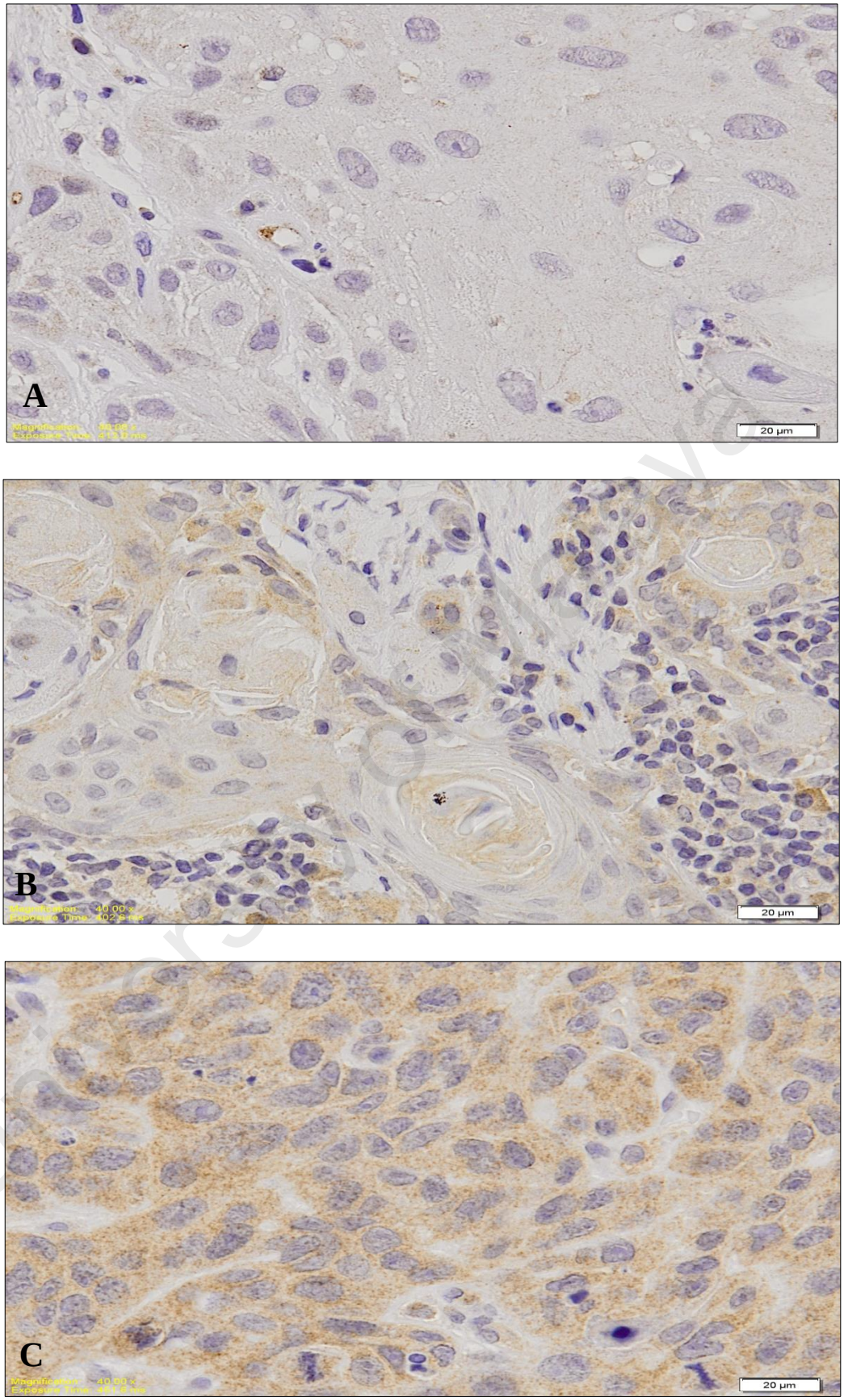

Figure 4.9 VEGF expression scoring.
(A) Negative staining. (B) Mild positive staining in OSCC. (C) Moderate positive staining in OSCC.

4.5 Association between regression grading and expressions of markers

For the purpose of statistical analysis, samples with podoplanin and VEGF staining scores of less than 4 (IRS score) were considered as low staining while scores of equal to or higher than 4 were considered as high staining (de Vicente et al., 2015). More than half of the tumours with strong staining of podoplanin (58.3%) in the biopsy samples prior to NAC were graded as having little or no regression after the treatment. There was no significant result obtained for the association between the regression grading and expression of both markers as shown in Table 4.7.

Table 4.7 Association between AJCC-TRG and podoplanin and VEGF expression

Characteristics	Value n (%)	AJCC-TRG, n (%)				*p**
		0	1	2	3	
• **Podoplanin**						
Low staining	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	2(100.0)	
High staining	12 (85.7)	2 (16.7)	2(16.7)	1 (8.3)	7 (58.3)	1.000
Total	14(100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	
• **VEGF**						
Low staining	6 (42.9)	0 (0.0)	0 (0.0)	0 (0.0)	6(100.0)	
High staining	8 (57.1)	2(25.0)	2 (25.0)	1(12.5)	3 (37.5)	0.147
Total	14(100.0)	2 (14.3)	2 (14.3)	1 (7.1)	9 (64.3)	

* : Test performed: Fisher's Exact test; Level of significance: $p<0.05$.

CHAPTER 5: DISCUSSION

Tumour regression grading (TRG) systems were meant to standardize histopathologic changes seen in tumours after neoadjuvant therapy, and categorize patients based on response to the treatment. Authors have used various grading systems to predict patient's response to the treatment, which usually included cytoplasmic vacuolization and/or eosinophilia, nuclear pyknosis, necrosis of the tumour cells, fibrosis, and presence of multinucleated giant cells surrounding ghost cells and keratin. In our study, we proceeded with the use of the three most common and well-established TRGs, namely the Mandard-TRG, Ryan-TRG and AJCC-TRG for the grading of our OSCC samples. Our results from the tumour regression gradings were very consistent for all three TRG systems. This was well-reflected by the interobserver measure of agreement which ranged from good with Mandard-TRG (Kappa=0.62), slightly better with AJCC-TRG (Kappa=0.74) and very good with Ryan TRG system (Kappa=1.00).

In the course of our tumour regression grading exercise, 9 (64.3%) out of 14 samples were found to have poor response and graded as Grade 3 with the Ryan-TRG and AJCC-TRG systems. Meanwhile with Mandard-TRG, the same 9 samples were divided between Grade 4 (5; 35.7%) and Grade 5 (4;28.6%). This proved crucial as the discrepancies of scores between the two examiners were mostly noted between TRG 4 and 5 as well as TRG 1 and 2 for the Mandard-TRG. The discrepancy between TRG 4 and 5 was similar to that reported in Ryan et al. (2005) and Chetty et al. (2012) with regards to the overall degree of fibrosis and necrosis as tumour response to NAC. When using the 4-point and 3-point TRG systems (AJCC & Ryan-TRG), the majority of the tumours were reproducibly stratified to the correct categories, with no disagreement found in Ryan-TRG, indicating that this 3-point TRG system had the advantage of better reproducibility, with similar prognostic significance when compared to 5-point Mandard-TRG system.

Our study also showed that 2 (14.3%) out of 14 tumour samples had complete tumour regression (pathologic complete response-PCR) which was equivalent to Grade 1 for Mandard-TRG and Grade 0 for AJCC-TRG system. We found it inconvenient that while AJCC-TRG had a grade specific for complete regression (Grade 0), the Ryan-TRG system was more indiscriminate as this feature was combined under Grade 1, together with moderate regression category. For reasons described above, we proceeded with the use of AJCC-TRG system to further assess the relation between PCR and the effectiveness of NAC treatment. Our decision was further reinforced by the fact that the AJCC-TRG system had been suggested to be adopted as the gold standard for grading rectal cancer response to NAC (Trankarnsanga et al., 2014; Mace et al., 2015) and was a proven significant prognosticator for recurrence and OS (Zhang et al 2016; Zhu et al 2017). Even though there have not been any studies to assess the effectiveness of AJCC-TRG system in grading tumour response in head and neck cancer or OSCC, the results from our study suggested that AJCC-TRG system was comparable to the other known TRG systems in terms of reproducibility of the grading and similarity of the histopathological criteria for assessment.

In Malaysia, the chemotherapy agents prescribed to OSCC patients in NAC treatment were either cisplatin with 5-Fluorouracil (PF) or PF combined with Docetaxel (TPF). TPF was the treatment of choice if the patient was young and without any comorbidity, while PF was usually given to older patients with other comorbidity, because it was less toxic compared to TPF. Incidentally, all of the patients in our study were administered with PF regimen. We found that about 64.3% of tumours in our samples showed little to no regression after NAC, in this case specifically the PF regimen. This implied that NAC as a treatment option was not particularly favourable and did not offer added benefit compared to primary surgery since most of the patients did not respond well to NAC in the first place. This finding was in concordance with other studies evaluating the role of

NAC as one of the treatment options for OSCC (Licitra et al., 2004; Joshi et al., 2013; Nanda & Mohiyuddin, 2015; Lau et al., 2016). However, these studies usually assessed the prognosis through parameters like overall survival and loco-regional control which were not covered in our research. Our patient pool was generally not mature enough (samples only available from 2012 till 2017) to produce data necessary to assess these parameters which may better reflect the effectiveness of the NAC. Limited number of samples and less-refined criteria in patient selection for NAC might have also affected our findings. Nevertheless, as most of the literature, including our research did not present strong findings in support of NAC, it would explain why NAC is currently not the mainstay treatment of OSCC and the reason NAC is not used more widely by clinicians in their management of OSCC despite its touted benefits (Licitra et al., 2004; Joshi et al., 2013; Nanda & Mohiyuddin, 2015; Lau et al., 2016).

When tumour regression grading was further analyzed against clinicopathological parameters, the only significant result that we managed to achieve in our study was the correlation between tumour regression grading and ypTumour size. Poor response to NAC was significantly correlated to larger tumour size ($p=0.001$). However, in reference to the other parameters such as advanced pTNM staging, positive surgical margin status, positive lymph nodes and extracapsular spread, and tumour differentiation, there was notably a strong association of these parameters with higher grade of TRG, which was indicative of poor response to NAC. These findings were consistent with Mace et al. (2015) who reported that poor response to NAC was significantly associated with ypStaging, positive lymph nodes metastases, and also tend to be associated with angiolymphatic invasion and positive margin. These observations might however be confounded by the fact that the study by Mace et al. (2015) was conducted on rectal carcinomas. On the other hand, the study done by Wedemeyer et al. (2014) which compared Mandard-TRG and Braun-TRG systems in oral cancer patients did find that

regression grading was significantly associated with at least lymph node metastasis and surgical margin status. Failure to show significant correlation with these parameters in our study might be due to our small sample size, which limited the possibility of achieving significant results.

Our study also examined the prognostic value of two biomarkers; podoplanin (PDPN) and vascular endothelial growth factor (VEGF) in OSCC patients. Both proteins have been investigated previously as prognosticators in OSCC, though not necessarily in OSCC patients treated with NAC.

PDPN staining in our tumour tissues demonstrated two distinct patterns of staining. One was diffuse cytoplasmic and membranous staining in most of the tumour cells. Second pattern showed focal strong expression at the periphery of the tumour islands with no expression in the central areas, especially at the invasive front. These findings were similar to other studies such as in Yuan et al. (2006); Atsumi et al., (2008); Margaritescu et al. (2010) and Kreppel et al. (2010 & 2011). Yuan et al. (2006) proposed that although the biologic functions of podoplanin in tumorigenesis were not fully understood, overexpression of podoplanin could promote the formation of elongated cell extensions and increase adhesion, migration and tube formation of vascular endothelial cells, suggesting a role in cytoskeletal reorganization. This could be the explanation for the stronger expression of PDPN found at the invasive front, where it may play a significant role in tumour migration and epithelial-mesenchymal transition (Margaritescu et al., 2010; de Vicente et al., 2015; Quintanilla et al., 2019).

From our results, we found that although not significant, high expression of PDPN was seen in more than half of the tumours with higher grade in AJCC-TRG system which translated to having little to no regression. This indicated that overexpression of PDPN was strongly related to poor response to NAC which was clearly in agreement with the

study done by Kreppel et al. (2011). They reported a significant correlation in high levels of PDPN expressions in their pretreatment biopsy samples with non-regression of the tumour and poor overall survival. They also suggested that PDPN might serve as a prognostic factor to predict treatment response to NAC as well as for overall survival and locoregional control. As discussed above, higher grade in AJCC-TRG system was strongly correlated to worse prognostic factors such as advanced pTNM staging, positive surgical margin, positive lymph node metastasis, extracapsular spread and poorly differentiated tumour. In light of this, we could also surmise that overexpression of PDPN in biopsy specimens was associated with poor prognosis of OSCC patients in addition to its potential in predicting poor response to NAC.

Our second marker, VEGF, also demonstrated similar and distinct patterns of expression in tumour cells as seen in PDPN expression. Surprisingly we did not observe expected strong expression of this marker in the tumour cells. Majority of our samples merely expressed moderate staining (57.1%). Similar findings on the pattern of expression for VEGF have been echoed by other authors as well (Shimada et al., 2002; Kim et al., 2015). VEGF is a type of angiogenic protein factor that can induce proliferation, differentiation and migration of vascular endothelial cells. Its receptors (VEGFR) such as VEGF-1 and VEGF-2 are mainly located in the cell membranes of endothelial cells and activated after binding to other factors in the extracellular matrix. They are also known to promote cell nucleus division and contribute to angiogenesis through extracellular dissolution and endothelial cell movement (Fontanini et al., 1997). In the present study, 85.7% of our tumour samples expressed a variable degree of VEGF staining. This finding was similar to the findings by Margaritescu et al. (2010) who claimed that 87% of their surgical tumours were positive for VEGF. Meanwhile Kim et al. (2015) also reported that all of their tumours exhibited VEGF expression with 45% of their samples having low-level staining and the remaining 55% demonstrated high-level

staining. They also found that VEGF expression was increased in the cytoplasm of invasive tumour fronts in moderately differentiated OSCC, and a remarkable increase was observed in poorly differentiated OSCC as compared to normal tissue and intraepithelial carcinoma tumour cells (Kim et al., 2015).

In our study, high and low expressions of VEGF were more or less equally distributed against every grade of AJCC-TRG. Hence, there was no appreciable correlation between VEGF expression with AJCC-TRG and by default, response to NAC. Therefore, VEGF did not appear to be a reliable predictor of treatment response to NAC. However, in contradictory findings by Martin et al. (2007), they suggested that the expression of VEGF in head and neck squamous cell carcinoma (mainly upper aerodigestive tract cancer) can be used to predict the outcome but after primary radiotherapy instead of NAC. The design of their study was almost similar to ours in that pre-treatment biopsy specimens were also stained with VEGF. They found that 5 years local control was observed in tumours with low-level of VEGF expression as compared to 18% of tumours with high level expression. The same results applied when they compared VEGF staining to the overall survival of the patients. The author also highlighted the potential role of VEGF expression in predicting resistance to the primary radiotherapy (Martin et al., 2007). To the best of our knowledge, apart from this study by Martin et al. (2007), VEGF has never really been assessed for its potential as a predictor of treatment response to NAC in oral squamous cell carcinoma.

VEGF was more frequently investigated for its role as a prognosticator in OSCC as evidenced by findings from studies such as Maeda et al (1998); Shimada et al (2002); Uehara et al. (2004); Margaristescu et al (2009 & 2010); Cheng et al. (2011) and Kim et al (2015). These studies mostly agreed that VEGF expression was highly correlated with poor prognosis in patients with OSCC. Cheng et al. (2011) in his study with 100 OSCC patients demonstrated that higher VEGF expression was significantly correlated with

positive lymph node metastasis and advanced clinical stage. In addition, Kyzas et al. (2005) in their meta-analysis study found that overexpression of VEGF seemed to be associated with worse overall survival in head and neck cancer. On the contrary, Margaritescu et al. (2010) found that low expression of VEGF tended to be associated with poorly differentiated OSCC in terms of tumour differentiation.

From the lack of association with AJCC-TRG, VEGF expression in our study seemed to harbour limited role as a prognosticator for OSCC. While our limited samples could be a decisive factor, we did however faced difficulties during the optimisation stage for this protein whereby multiple troubleshooting sessions had to be carried out with the supplier. Perhaps there was a slight possibility that this batch of antibodies produced suboptimal results.

CHAPTER 6: CONCLUSION

In conclusion, we found that NAC as a treatment option for OSCC was less than favorable with no supplementary benefit when compared to the principal surgical treatment option alone. In addition, tumour regression grading remained a good indicator in predicting tumour response to NAC in OSCC as it correlated well with most of the clinicopathological features of OSCC. We would like to propose the use of AJCC-TRG system as a suitable and relevant tumour regression grading system for head and neck cancer, especially OSCC. In terms of biomarkers, podoplanin showed great promise as a prognosticator for OSCC as its overexpression was correlated with larger tumour size, advanced stage of tumour, positive involved surgical margins, lymph node metastasis and extracapsular spread. Podoplanin also exhibited great potential as a predictor of tumour response to NAC. VEGF, on the other hand, was neither promising as a predictor of tumour response to NAC nor a reliable prognosticator for OSCC.

The limitations in our study were mainly due to an unexpectedly small sample size, as a remarkable number of patients who underwent NAC, would either drop out of the treatment midway, or could not complete the treatment until the surgical stage. Apart from that, our overall data was understandably immature thus preventing data analysis with the definitive outcomes of cancer treatment such as overall survival (OS), locoregional failure, disease-free survival and etcetera. Thus, this study could only serve as an early assessment for the efficacy of the NAC in OSCC patients in Malaysia.

We recommend continued and followed up studies using the AJCC-TRG system and podoplanin expression with a bigger sample size in future.

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