# Prognostic Significance of Epithelial to Mesenchymal Transitions-Related Markers in Oral Squamous Cell Carcinoma

**Chimi Wangmo**

**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Health Sciences**
**Prince of Songkla University**
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| **Major Program** | Health Science |

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(Prof. Dr. Damrongsak Faroongsarng)
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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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## ABSTRACT

**Objective:** We aimed to evaluate the association between the expression of E-cadherin and vimentin with clinicopathological characteristics and 5-year overall survival in oral squamous cell carcinoma (OSCC).

**Material and methods:** A total of 200 surgically resected OSCC patients treated during 2008-2011 were included. The protein expression analysis was performed using immunohistochemical technique on paraffin-embedded microarray tissue slides Immunoreactivity of proteins were classified into two groups using score of intensity multiplied by percentage of positive tumor cells. A combined analysis was performed using both E-cadherin and vimentin expression to determine Epithelial to mesenchymal transition (EMT) status. Kaplan-Meier method and log-rank test were used to compare the differences among survival curves. Cox-regression analysis was used to obtain independent prognostic factors.

**Results:** The median survival time was 48 months. Twenty-eight (14%) tumors showed loss of E- cadherin expression and 172 (86%) showed preserved E-cadherin expression. Vimentin was negative in 113 (56.5%) tumors and positive in 87(43.5%). E-cadherin (P=0.008) and EMT status (P=0.02) were significantly associated with lymph node metastasis. E-cadherin and vimentin were significantly associated with 5- year overall survival with HR 1.94, 95%CI (1.19-3.16) and HR 1.85, 95% CI (1.26-2.7) respectively. EMT status was also significantly associated with 5-year overall survival, the hazard ratio increased with EMT progression. Complete EMT (loss of E-cadherin expression and positive vimentin) status had higher hazard ratio then individual proteins (HR 2.88, 95% CI (1.44-5.79)).

**Conclusion:** Though both E-cadherin and vimentin expression could act as an independent prognostic factor, a combined evaluation of E-cadherin and vimentin expression to evaluate EMT status could provide a stronger indicator of prognosis in OSCC in addition to age, stage and treatment modality in surgically resected oral squamous cell carcinoma.

## ACKNOWLEDGMENT

I would like to express my deepest gratitude to my advisor Associate professor Paramee Thongsuksai. Without her guidance, encouragement and constructive criticism this research would not have been possible.

I would also like to thank all the laboratory staffs in Pathology department for helping me with laboratory work. Without their assistance my work won't have been complete.

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## List of abbreviations

AJCC: American Joint Committee of Cancer

ASR: Age standardized rate

CMT: Chemotherapy

EMT: Epithelial to mesenchymal transition

HNSCC: Head and neck squamous cell carcinoma

LVSI: Lymphatic and vascular invasion

N: Cervical nodal metastasis

OSCC: Oral squamous cell carcinoma

PNI: Perineural invasion

RT: Radiotherapy

T: Size as per TNM staging

# Chapter 1

# Introduction

## 1.1. Background and rationale

Oral cancer is 11th most common malignancy worldwide with ASR of 7.0 with 300,286 cases being reported in 2012, of which majority of case 103,464 (34%) were in South East Asia (1). In Thailand, it is the 11th most common cancer with an ASR 4.0 per 100,000 (2). Oral cavity cancer is commoner in the southern region. The age-standardized incidence rate (ASR) of oral cavity cancer in males in Songkhla province, is higher compared to other regions in Thailand (incidence 9.1 per 100,000)(3).

Oral squamous cell carcinoma comprises of more than of all oral cancers are treated by surgery, radiation, and adjunct chemotherapy either in combination or separately. Nonetheless, the five-year survival rate is poor at about 50% with recurrence and distant metastasis occurring in more than one third of treated patients (4, 5). Furthermore, surgical treatment causes facial disfigurement, and functional disturbances during mastication and speaking. Poor survival in oral cancer could be credited to the higher rate of recurrence and metastasis.

Epithelial-mesenchymal transition (EMT) is a process that enables polarized, immotile epithelial cells to convert to motile mesenchymal cells and is characterized by the combined loss of epithelial cell junction proteins such as E-cadherin and the acquisition of mesenchymal protein such as vimentin. E-cadherin is the main molecule in adhering junctions of epithelial cells while vimentin is a mesenchymal protein associated with migratory phenotype (6). Nijkamp et al. (7) reported that loss of expression of E-cadherin and gain of vimentin expression to be correlated with metastasis formation in head and neck squamous cell carcinoma patients.

EMT plays crucial roles in embryonic development via enabling differentiation of organs, however in setting of carcinogenesis EMT confers an invasive phenotype to cancer cell and act as a crucial regulator of metastasis and invasion (8, 9). EMT also confers cancer stem cell properties (10) and has been reported to be responsible for resistance to chemotherapy and immunotherapy (11).

Previous studies have reported immunohistochemical expression of E-cadherin and vimentin in OSCC (12-14) but results regarding its prognostic significance are

limited and controversial. Furthermore, none of the previous study have evaluated E-cadherin and vimentin expression combined together to evaluate EMT status in OSCC.

We aim to study immunohistochemical expression of E-cadherin and vimentin individually and combined (EMT status) to see their association with clinicopathological characteristics and its prognostic significance in OSCC.

### 1.2. Research Question

Is immunohistochemical expression of E cadherin and vimentin (individually and combined to represent EMT status) associated with clinicopathological variables and survival in OSCC?

### 1.3. Hypothesis

Loss of E-cadherin expression and gain of vimentin expression in oral cancer leads to poorer prognosis in oral cancer

### 1.4. Objectives

To determine the association between immunohistochemical expression of E cadherin and vimentin individually and combined (EMT status) with clinicopathological characteristics and 5-year overall survival in surgically treated OSCC

### 1.5. Literature review

#### 1.5.1. Oral squamous cell carcinoma

More than 90% of cancers in oral cavity are oral squamous cell carcinoma (OSCC). It frequently occurs in fifth and sixth decades of life.

Oral cancer along with oropharyngeal cancer is the sixth most common cancer in the world with age standardized incidence rate of 4.0 and a mortality rate of 1.9 (15). 300,373 new cases were estimated in 2012 by GLOBOCAN project. Oral cavity cancer is commoner in the southern region. The age-standardized incidence rate (ASR) of oral cavity cancer in males in Songkhla province, is higher compared to other regions in

Thailand (incidence 9.1 per 100,000)(3), which is higher than global incidence. According to 2015 hospital cancer registry, OSCC is the fourth most common cancer in Songklanagarind hospital.

Smoking is one of the prime causes of oral cancer (16). Other alcohol consumption and betel quid chewing also increases the risk of oral cancer (17, 18). Though HPV has been reported as risk factor in oropharyngeal cancer, in OSSC only a small portion (3%) shows HPV positivity (19).

Tongue is the most common site for oral cancer followed by floor of the mouth and gingiva. The clinical features ranges from asymptomatic form to ulceroproliferative growth depending on stage at presentation and location. Cancer of the tongue appear as a red area with nodule or ulcer while tumors of buccal mucosa and lips usually present as an ulcer. Unlike other area tumors of the hard palate often present as papillary or exophytic growths.

Grossly oral cancers are firm irregular with gray white or tan cut surface. Histologically most of the squamous cell carcinoma are well or moderately differentiated; poorly differentiated tumor is less frequent.

OSCC are mainly treated by surgery unless in cases where the patients are unfit for surgery in which case they are treated with primary radiotherapy and adjunct chemotherapy (20). There has been a decrease in trend of nonsurgical treatment for OSCC as per the study conducted in United States by Fujiwara where they had included all oral cancer patients in National Cancer Data Base from 1998 to 2011 (21).

### 1.5.2. Prognostic factors in OSCC

OSCC has predisposition for local invasion and early lymph node metastasis. As per the WHO classification of head and neck tumors the most significant prognostic factors in oral cancer are the tumor size, lymph node metastasis status and distant metastasis.

Tumor size determines the surgeon's ability to resect tumors with free margins (22) and determines radiotherapy dose for treatment (23). In study by Moore et al where they analyzed correlation between tumor size and lymph node metastasis, tumors < 2 cm correlated with fewer lymph node metastases compared to tumors > 2 cm (24).

Lymph node and distant metastasis are associated with adverse outcome in OSCC. Ho et a. reported that the number of lymph node metastasis to be more critical indicator of survival compared to nodal metastasis size or contra laterality of nodal metastasis (25). Lieu et al. found all level IV/V metastases to have the poorest prognosis which was further worsened when extracapsular invasion was present (26).

Distant metastasis in oral cancer ranges from 3.8 to 9.6% and commonly involves lung, bone and liver (27, 28). The majority of distant metastasis occur within 2 years after the initial therapy, and most patients die within a year after the diagnosis (27).

Posteriorly located tumors are more aggressive compared to anteriorly located tumors in OSCC. In study by Woolgar et al. higher proportion of patients with retromolar (38%) and oropharyngeal (41%) and had died compared to patients with floor-of-mouth (10%) and buccal tumors (17%).

In study by Sutton et al. fewer number of patients (11%) with an involved margin were alive compared those with close (47%) and clear margins (78%) (29). Lymphovascular invasion, perineural and bone invasion are also associated with prognosis (30, 31).

HPV is a predominant etiological factor in oropharyngeal cancer however only a small number of OSCC show HPV positivity. In study by Lingen et al of 409 OSCC cases, only 24 (5.9%) were HPV positive (32), similarly HPV was detected in only 2 (3.2%) out of 62 OSCC in study by Thomas et al (33).

In study conducted by Fujiwara et al. in United States, where they had included all oral cancer patients in National Cancer Data Base from 1998 to 2011, they found that non-surgical treatment to be associated with decreased overall survival in both early and late stage (21). Similarly, primary radiotherapy for early stage OSCC were reported to be associated with increased mortality in study by Ellis et al. (34).

### 1.5.3. Epithelial to mesenchymal transition

The proposition that epithelial cells downregulates epithelial characteristics and acquire mesenchymal characteristics was first reported in 1982 by Greenburg and Hay (35). The term epithelial–mesenchymal transition (EMT) highlights its transient nature with the reverse process described as mesenchymal–epithelial transition (MET). It is

considered complete EMT if there is complete loss of epithelial traits and complete gain of mesenchymal traits and partial EMT when there is partial loss of epithelial traits with partial gain of mesenchymal traits.

Epithelial cells are held together with adjacent cells and to the underlying basement membrane in polarized sheets by various cell adhesion molecules, such as E-cadherin and claudins. These adhesions are critical for maintaining the epithelial phenotype. During EMT loss of adhesion molecules triggers change in cytoskeletal architecture which alters the cell polarity to form a migratory mesenchymal cell with gain of mesenchymal proteins such as vimentin and α-smooth muscle actin which enable them to invade basement membrane and underlying tissues. Loss of expression or function of adhering junctions such as E-cadherin and concomitant increase mesenchymal proteins such vimentin is considered hallmarks of EMT (Figure 1) (36).

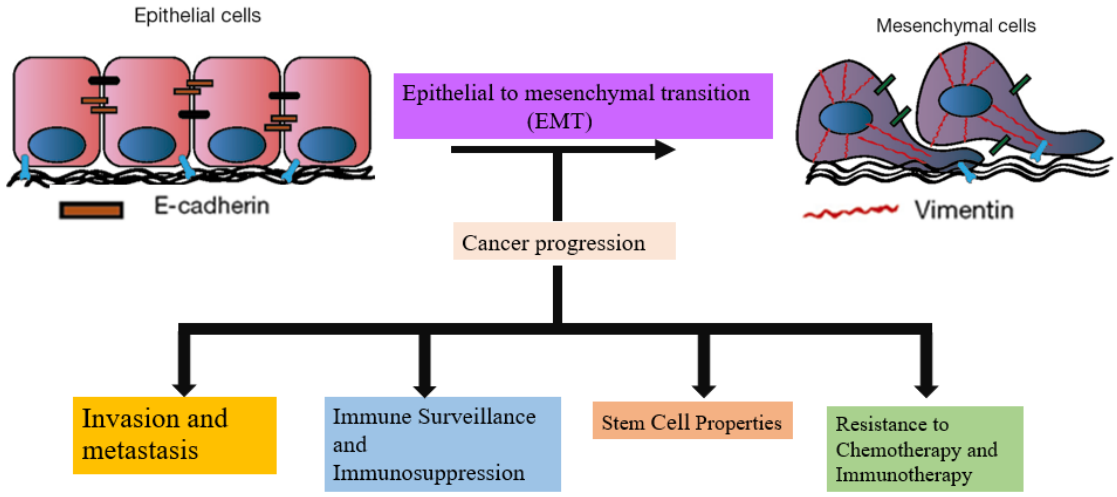


**Figure 1**: **Epithelial to mesenchymal transition**(37)

The epithelial cells that acquire mesenchymal phenotype develop resistance to apoptosis and gain stem cell like characteristics. In non-carcinogenic setting of embryogenesis and wound healing, these characteristics of EMT allows the developing normal cell to migrate and resist apoptosis. However, in the setting of cancer, EMT enables the development of an invasive and migratory phenotype (8, 38).

Though the full spectrum of signaling agents that induce EMT is unknown, in the setting of carcinogenesis EMT inducing signals originating from the tumor associated stroma growth factors such as TGF-β, EGF, PDGF and HGF appears to be

responsible for activation EMT transcriptional factors (Figure 2) such as Twist, Snail, Slug, ZEB1 and FOXC2 (5).

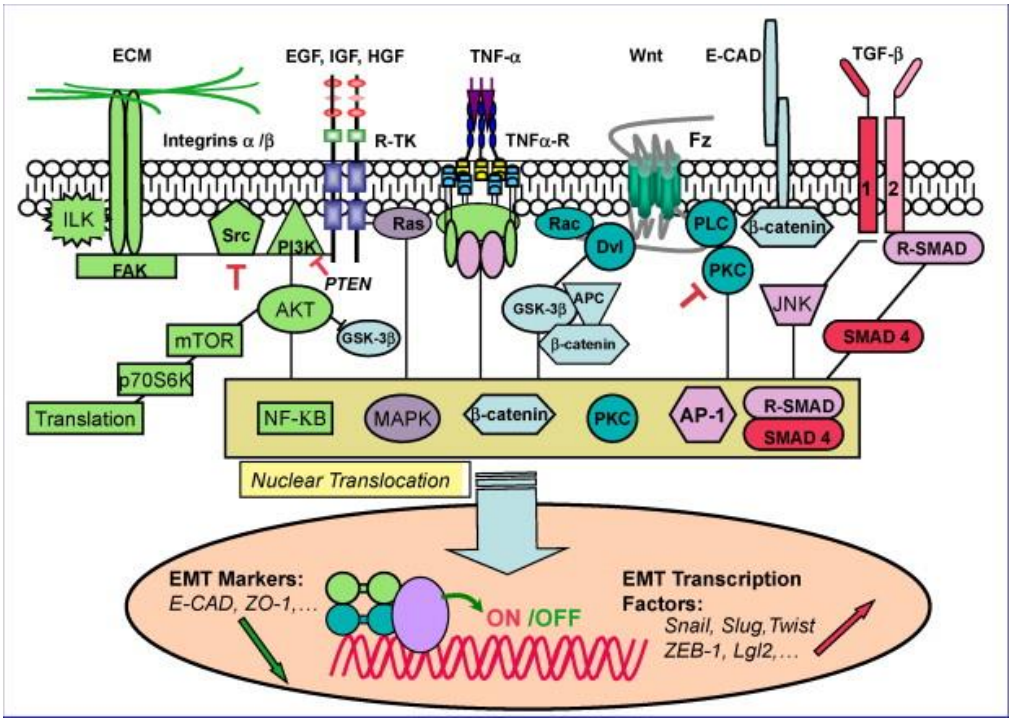


**Figure 2:Molecular mechanisms regulating EMT** *(39)*

Other than playing role in metastasis, EMT enhances cancer progression by suppressing immune system (40), conferring stem cell properties (41) and augmenting resistance to radio and chemotherapy (42).

Snail was shown to hasten cancer metastasis through enhanced invasion and immunosuppression in murine and human melanoma cells (43). In study by Noman et al. human breast cancer cells showed upregulation of immune checkpoint ligand PD-L1 by a mechanism involving ZEB-1, a transcription factor in EMT (40).

In mammary epithelial cells expression of SNAIL or TWIST induced a mesenchymal cell population with stem cells properties marked with CD44 high/CD24 low phenotype (41). In study by Ota et al where cancer stem cell properties were assessed using sphere formation and WST-8 assays, Snail-induced EMT was seen to promote cancer stem cell-like properties in head and neck cancer cells (44).

In experiment conducted by Fischer et al. in mice, cells with EMT phenotype resisted cyclophosphamide treatment (45). In breast cancer cell line expression of

Twist, a transcription factor in EMT, lead to increased migration, invasion, and resistance to Paclitaxel (46).

As EMT plays a substantial role in all the major carcinogenesis phases, exploring and understanding EMT further will unequivocally enhance the chances of better treatment for cancer and improve survival.

### 1.5.4. E-cadherin and vimentin

A vast range of proteins play role in EMT, the predominant ones could be categorized as follows: cell-surface consisting of E-cadherin, N-cadherin, and Integrins; cytoskeletal protein consisting of vimentin, α-Smooth Muscle Actin and β-catenin; extracellular matrix protein consisting of collagens, fibronectin, and laminin and transcription factors consisting of SNAIL1, SNAIL2, TWIST, and LEF-1. In head and neck cancer studies low expression of E cadherin and high vimentin expression are reported as EMT markers (47, 48). Liu et al. (49) demonstrated that down-regulation of microRNA-138, induced mesenchymal-like cell morphology and enhanced cell migration and invasion accompanied by marked reduction in E-cadherin expression and enhanced vimentin expression in head and neck cancer cell line. Nijkamp et al. (7) also found loss of E-cadherin and gain of vimentin to enhanced migration of tumor cells, leading to higher metastatic in HNSCC patients. Liu et al. (50) reported vimentin as a potential prognostic factor among other EMT related proteins including Snail, Twist, E-cadherin, and N- cadherin for tongue squamous cell carcinoma.

E-cadherin is a fundamental protein of adherens junctions that anchor oral epithelial cells to adjacent cell and basement membrane. It is a calcium-dependent cell-surface protein that promote adhesion of epithelial cells to adjacent cell. E-cadherin constitutes two domains, the intracellular and extracellular domains that create homophilic interactions between neighboring cells to enhance adhesion. The intracellular domain of E-cadherin is linked to the actin cytoskeleton through its interaction with its cytoplasmic-binding partners, the catenin family, namely β-catenin and p120-catenin.

During carcinogenesis there is loss or reduced function of E-cadherin which debilities cell-cell adhesion and releases β-catenin. β-catenin translocates to the nucleus and in turn induces transcription genes of EMT like TWIST and Snail (51). Reduced

E-cadherin expression is associated with poorer prognosis in cancers of esophagus (52), stomach (53) and ovary (54).

As loss of E-cadherin are indicative of EMT and cancer progression, studies have found that E-cadherin could help to decide the best treatment modality in cancer patients. Patients with higher E-cadherin expression has been reported to have better sensitivity toward the EGFR-tyrosine kinase inhibitors (55).

Vimentin is an integral part of the intermediate filament family of proteins. Vimentin are expressed by mesenchymal cells like fibroblasts and endothelial cell and not expressed in normal epithelial cells. Therefore, they are used as a marker of mesenchymal cells to distinguish them from epithelial cells.

Vimentin expression is associated with a migratory phenotype and resistance against stress (56). Vimentin mediate cytoskeleton architecture change in cancer cells and leads to resistance against stress by forming polarized microtubules at the juxtanuclear region and forming stress fiber actin structures at the cell bottom. Actin filaments forms spike-like structures at the cell periphery leading to cell membrane extension thereby increasing the mechanical strength of the cells and the ability for cell migration as shown in Figure 3.

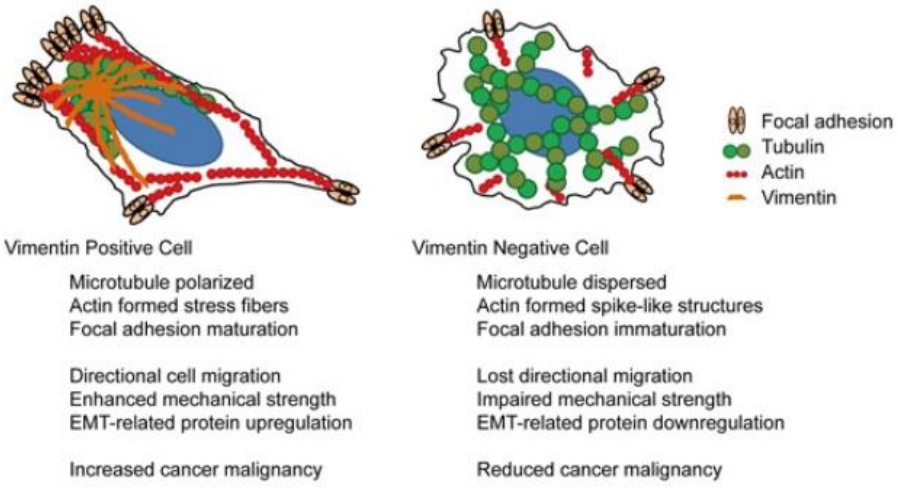


**Figure 3: Role of vimentin in EMT-related mechanoregulation*(57)***

Vimentin expression has been reported to be associated with tumor invasion and a poor prognosis in cancers of breast, colorectal carcinoma and lung cancer (58-60).

Vimentin has been reported to be expressed in head and neck squamous cancer cell line (61). Vimentin expression in head and neck cancer are induced by stromal growth factors like epidermal growth factor and TGF-β (62). Due to its role in EMT, anti-vimentin drugs are being explored. A drug called Withaferin-A has been shown to promote degradation of vimentin resulting in inhibition of cell migration and invasion in vitro experiments (63).

Multiple previous studies have explored expression of E-cadherin and vimentin. However, there are limited studies that have evaluated its prognostic significance with contradictory results. Liu et al showed vimentin and E-cadherin expression in 83 OSCC to be associated with overall survival (RR 1.612, 95% CI 1.017-2.554, P 0.042 and RR 0.579, 95% CI 0.372- 0.903, P 0.016) respectively and as an independent prognostic factor. Fan et al also reported E cadherin expression to independent marker for survival prediction in OSCC (HR 0.41, 95% CI 0.22–0.75, P 0.004). Contrary to the above study, Costa et al. and Balasundarm found no association of E cadherin with outcome in their studies. Vimentin expression significantly correlated with survival (P = 0.021) in 227 OSCC patients in study by Sawant et al. (14). However, other studies that explored vimentin expression found no association with outcome in OSCC (64, 65). Table 1 summarizes the above studies.

No previous study has evaluated the combined prognostic significance of E-cadherin and vimentin, that is EMT status, in OSCC. Aruga et al. who evaluated EMT phenotype in lung squamous cell carcinoma by combining E-cadherin and vimentin also found EMT phenotype to be a significant indicator of poor prognosis in lung squamous cell carcinoma (66).

**Table 1: Summary of previous studies of E-cadherin and vimentin in OSCC**

| Sources | Cases | Antibody /scoring | Result | Remarks |
|---|---|---|---|---|
| Zhou et al. (67) *China* | 42 OSCC | Mouse anti-human monoclonal vimentin and E-cadherin<br>Ab Scoring = intensity × positive cell. | 24(61.9%) expressed E cadherin and 16 (38%) vimentin and associated with lymph node metastasis<br>E-cadherin was also associated with tumor stage | Less number of patients<br><br>Have not evaluated prognostic significance |
| Liu et al (12) *China* | 83 OSCC | E-cadherin mouse monoclonal ab<br>Vimentin rabbit monoclonal ab<br>Immunoreactivity score=intensity score × proportion score . | 23 (53%) expressed vimentin and associated with recurrence and death<br>Decreased E-cadherin in 43 (84%) associated with recurrence and death<br>Vimentin RR 1.612 CI (1.017, 2.554)<br>E-cadherin RR 0.579 CI (0.372, 0.903) | Less sample sizes<br><br>No combined analysis |
| Costa et al**.** 2015(64) *Brazil* | 20 OSCC | E cadherin and vimentin monoclonal antibody.<br>E-cadherin classified "preserved" - > 50%, reduced"- < 50%.<br>Vimentin classified "negative" < 10% "positive" >10% | 15 (75%) had reduced E-cadherin, expression was reduced at the invasive font (ID) compared to the central or superficial area (CSA).<br>Vimentin was positive in 6 (30%) no difference between the IF and the CSA regions. No correlations with tumor stage or nodal status | Less sample size<br>Subsite not mentioned<br>Have not analyzed prognostic significance |
| Balasundra et al**.** 2014(68) *India* | 60 OSCC 30 with and 30 without | E-cadherin and vimentin primary antibodies | All cases expressed vimentin and E cadherin | Less sample<br>Only 2 subsites included |

| | | | | |
|---|---|---|---|---|
| | lymph node met | Immunoreactivity score = intensity score x proportion score . | No significant difference between E-cadherin and vimentin expression in OSCC with and without lymph node metastases . | Prognostic significance not evaluated |
| Siliva et al. 2015 (65)<br><br>*Canada* | 102 OSCC | Anti-mouse E-cadherin and vimentin Ab | Patients with multiple primary tumor had lower survival (P<0.0001), for tumors showing negative protein expression for E-cadherin (P = 0.003) Vimentin were not able to predict the survival | Have included only cases with multiple primary OSCC No combined analysis |
| Fan et al. 2013(69)<br><br>*Taiwan* | 112 OSCC | Monoclonal E-cadherin<br>The staining intensity was estimated by its extent and intensity | Patients with lower E-cadherin expression had a poorer survival (40/66) than those with higher E-cadherin expression (46/10). E-cadherin associated with tumor location (P=0.04) and mortality (P=0.010) | Only E-cadherin evaluated |
| Freitas et al, 2006(70)<br>*Spain* | 47 OSCC | Primary anti e cadherin<br>Immunoreactivity assessed by staining intensity and percentage | Weak or absent E-cadherin associated with a more invasive histological pattern (P=0.004) shorter disease-free period (P=0.0014) and shorter survival time (P=0.0013) | Less sample sizes<br>Only E-cadherin evaluated |
| Sawant et al (14)<br>*India* | 227 OSCC | Anti-vimentin monoclonal antibody | Vimentin expression was associated with tumor size (P=0.048) clinical stage, | Only vimentin evaluated |

| | | | | |
|---|---|---|---|---|
| | | IHC staining was quantified by counting a total of 100 cells per field at 200× | (P=0.0013) regional lymph node metastases (P=0.001), local recurrence (P=0.001) survival (P=0.021) | |

### 1.5.5. Tissue microarray

Tissue microarray, a recent innovation in pathology allows analysis of numerous tissue specimen at the same time. A microarray contains small representative tissue samples assembled on a single histologic slide from different cases. Up to 1000 samples can be analyzed at the same time.

Principle**:** Tissue microarrays are made by drawing cylindrical tissue cores from different paraffin donor blocks and re**-**embedding them into a single recipient microarray block which allows for all samples to be analyzed at the same time.

Advantage and limitation of TMA and TMA validation studies**:**

The ability to simultaneously use of a large number of cases in a single slide give the great advantage of TMA technique regarding the standardization of the reaction of immunohistochemistry, a significantly reduced reagent volume and experimental handling time. In cases of heterogenous tumor cells a core of tissue may not act as a representative for the whole tumor. To evaluate this issue, studies on various cancer sites have been performed to determine the optimum size of the cores as well as the number of tissue cores to represent to whole tissue section. Most reported 0.6-2.0 mm core and 1-2 tissue cores are acceptable with moderate to high agreement with whole tissue slide (71-73). In OSCC, Chen et al 2003 (74) validated 0.6 mm cores (triplicate) in 184 HNSCC and found Kappa of 0.66 for cyclinD1, 0.40 for EGFR and 0.41 for Rb. Ramanathan et al, compared p53 expression of 3-6 virtual cores of size 0.6 mm, 1.0 mm and 1.5 mm drawn on the scanned slides of OSCC and found a good correlation (r = 0.826) with just a single core of 0.6mm (0.826) even though increase in core number and size (1.5 mm) resulted in improved correlation coefficient and smaller confidence interval. Monteiro et al (75), also demonstrated a high concordance of EGFR expression in OSCC in dual 1.5 mm core TMA with whole tissue sections (kappa

= 0.720). In this study, we used 2 mm cores for each case. As the previous evidence, it could be a good representation of whole tissue section.

# Chapter 2

# Research Methodology

## 2.1. Study design and targeted population

We conducted a retrospective cohort study in Department of Pathology, Songklanagarind Hospital. We targeted patients with squamous cell carcinoma of oral cavity including tongue, buccal mucosa, gingiva, lips, palate, floor of mouth subsites. We included surgically resected primary oral squamous cell carcinoma who were diagnosed and treated in Songklanagarind hospital during January 2008 and December 2011. Patients who had previously received chemotherapy or radiation therapy and cases with inadequate paraffin block or tissue for immunohistochemistry staining were excluded.

## 2.2. Sample size calculation

Sample size was calculated using the formula below which allows for testing a difference in the disease hazard with unequal samples. 5% significance and 80% power were used. Previous studies that analyzed survival for E cadherin and vimentin expression were reviewed (Table 2) to find hazard related to respective protein expression. Total sample needed ranged from 111 to 203, we concluded that about 200 cases would be required for our study.

$$n = \frac{\{Z_{\alpha/2}\sqrt{(1+k)g(\bar{\lambda})} + Z_{\beta}\sqrt{[k * g(\lambda_1) + g(\lambda_2)]}\}^2}{k(\lambda_1 - \lambda_2)^2}$$

$n$ = Sample size

$$\bar{\lambda} = [\lambda_1 + k\lambda_2]/(1+k)$$

$$g(\lambda) = \frac{\lambda^3 T_1}{\lambda T_1 - e^{-\lambda(T-T1)} + e^{-\lambda T}}$$

$Z_{\alpha/2}$ = 1.96 (confidence level 95%)

$z\beta$ = 0.84 (power 80%)

$\lambda 1$ = Hazard of positive of expressed group

$\lambda 2$ = Hazard of negative expression group

T = Time of follow up

$T_1$ = Time of enrollment

**Table 2: Sample size needed from literature review**

| **Author, year** | **Marker** | **No of cases** | **Marker** -/+) (**no** | **5-year OS** % (-/+) | **Total sample needed** |
|---|---|---|---|---|---|
| Freitas et al,2006 | E cadherin | 47 | 33/14 | 40/20 | 203 |
| Zhao et al,2012 | E cadherin | 98 | 76/22 | 50/25 | 180 |
| Fan et al, 2013 | E cadherin | 112 | 46/66 | 63/39 | 187 |
| Liu et al, 2012 | Vimentin | 83 | 57/26 | 30/62 | 111 |

## 2.3. Material and method

### 2.3.1. Patient and tissue samples

Two hundred cases of surgically resected primary OSCC that were diagnosed and treated in Songklanagarind hospital between January 2008 to December 2011 included. All of these cases had surgery as their first treatment and had undergone adjuvant radiotherapy and chemotherapy after surgery.

Records of follow up information were collected from hospital-based cancer registry. Clinical data including gender, age, alcohol, smoking and betel nut consuming habits, tumor sub-sites, tumor size and TNM staging were obtained from Hospital medical records.

Formalin-fixed, paraffin-embedded tissue samples and slides of corresponding cases of primary oral squamous cell carcinoma were retrieved from archives of Department of pathology. The study was performed with approval of the Human Research Ethics Committee of Songklanagarind Hospital.

### 2.3.2. Histopathological Evaluation

All slides were reevaluated**.** Pathological findings including tumor differentiation, margin adequacy, lymphatic and vascular invasion, nodal metastatic status and surrounding bone invasion status were reevaluated to confirm previous finding and were staged as per the World Health Organization classification 2017. Tumors were staged as per AJCC 2010.

### 2.3.3. Tissue microarray construction

Unitma (Seoul, Korea) tissue microarrayer were used for tissue microarray construction. All hematoxylin and eosin stained slides of oral squamous cell carcinoma were reviewed and two slides with representative tumor were selected from each case. One area of tumor stroma interface was circled on each slide. The area corresponding to the select area on the slide were circled on the formalin-fixed, paraffin-embedded block with a felt marker. Two cores from each case were cored out with a 2-mm diameter needle and transferred to a recipient paraffin block for tissue microarray

construction. After construction, 3-μm-thick sections of the TMA blocks were cut and stained with hematoxylin and eosin stain to assess adequacy and to ensure that the cores were representative of tumor.

### 2.3.4. Immunohistochemistry

The avidin-biotin method was used for immunostaining. The block was sectioned at 3-μm and transferred to glass slides. Each of these unstained sections were deparaffinized with xylene and rehydrated through a series of graded alcohol. Sections were stained with the antibodies, including monoclonal mouse anti-human E cadherin antibody (dilution 1:500, clone NCH-38, M3612, Dako, Denmark**)** and monoclonal mouse anti-vimentin antibody (dilution 1:100, clone V9, M0725, Dako, Denmark). Staining were performed with the Leica BOND-MAX automated immunostainer. The slides were incubated with peroxidase-blocking reagent, followed by the primary antibody then the visualization reagent using bond polymer refine detection kit. After that, the slides were incubated with 3,3-diaminobenzidine as a chromogen and counterstained by Mayer hematoxylin**.**

### 2.3.5. Evaluation of Immunoreactivity

All sections were examined by two observers separately without prior knowledge of the clinical data and outcome. Then all cases were jointly reassessed using multi headed microscope and cases with discrepancies were discussed until a consensus was reached.

The proteins expression was quantified by visual assessment under light microscopic, we first scanned using low power 40× magnification. and then evaluated the reactivity under 100× magnification. Staining intensity and the proportion of reactive tumors cells were assessed. The intensity was graded as 0 for negative, 1 for weak, 2 for moderate and 3 for strongest intensity. The proportion of reactive cells was graded as percentage ranging from 0-100%. The immunoreactivity was calculated by multiply intensity score with percentage. Each core was evaluated separately and a final score for each case was achieved by averaging the total immunoreactivity values of both cores.

### 2.3.6. Statistical analysis

Clinicopathological characteristics of the patients were presented in percent, mean and median and compared using chi- square tests or Fisher' s exact tests as appropriate. The association between E- cadherin and vimentin were analyzed using Spearman correlation coefficient and the categorized group by chi-square test.

The five-overall survival time was calculated from date of pathological diagnosis till the date of death or date of last follow up (June 2016). Patient who were alive at last date of follow-up and patient who had died from other cause rather than cancer were censored.

The Kaplan- Meier method were used to estimate the over survival ( OS) distributions, and the log- rank test were performed to compare the survival difference in each group. Univariate analysis was performed with log- rank test and by cox-regression. Multivariate Cox regression models were used to evaluate independent prognostic factor. Difference were considered significant when the P value <0. 05. Statistical analysis was performed by R program version 1.0.

The multiplied immunoreactivity ranged from 0 – 300. For statistical analysis, E- cadherin and vimentin were classified grouped into two groups, guided by log rank test and Kaplan Meier estimator and divided as follows: <= 60 loss of E-cadherin expression, > 60 as preserved E-cadherin expression, < = 10 as negative vimentin expression and >10 as positive vimentin expression.

# Chapter 3

# Results

## 3.1. Clinicopathological characteristics

Eight hundred and ninety-four cases of oral cancer were diagnosed in Songklanagarind hospital between 2008 to 2011. Only 281 cases were treated by surgery with or without adjunct radiotherapy and chemotherapy in Songklanagarind hospital. As we excluded cases with prior radiotherapy and chemotherapy and cases with missing paraffin block, we were left with 200 cases.

Table 3 summarizes the clinicopathological findings of our cases. The age ranged from 24-88 years with mean age of 61 years. One hundred and twenty-seven (63.5%) of the patients were male while 73(36.5%) were female. As we conducted retrospective study and obtained clinical details from hospital records few information regarding smoking, alcohol and betel consumption habits were not recorded in full details. Therefore, we classified them as smoker and betel consumer if they had ever consumed betel nut and smoked.

Tongue was the most common location comprising of 47.5% of the total case, followed by floor of mouth (18%) and buccal mucosa (11%). Most of the tumor were well differentiated (73.5%) and more than half of the cases (56%) were in stage III and stage IV.

All our 200 cases were surgically resected, of which 100 (50%) had received adjunct radiotherapy and 33 (16.5%) patients had adjunct radiotherapy with chemotherapy after surgery. Sixty-seven (33.5%) received surgical resection alone without adjunctive treatment. None of the cases were treated with adjunct chemotherapy alone.

**Table 3: Clinicopathological characteristics of patients**

| **Variables** | **No**. (%) | **Variables** | **No**. (%) |
|---|---|---|---|
| **Age** | | **Differentiation** | |
| Range (Mean) | 24-88 (61.2) | Well | 147 (73.5) |
| **Gender** | | Moderate | 47 (23.5) |
| Female | 73 (36.5) | Poor | 6 (3) |
| Male | 127 (63.5) | **T stage** | |
| **Smoking status** | | T1 | 65 (32.5) |
| No | 68 (34) | T2 | 64 (32) |
| Yes (ever) | 108 (54) | T3 | 19 (9.5) |
| Not available | 24 (12) | T4 | 52 (26) |
| **Alcohol drinker** | | **N stage** | |
| No | 79 (39.5) | N0 | 132 (66) |
| Yes | 80 (40) | N1 | 29 (14.5) |
| Social drinker | 9 (4.5) | N2 | 39 (19.5) |
| Not available | 32 (16) | **LVSI** | |
| **Betel nut** | | Not seen | 185 (92.5) |
| No | 73 (36.5) | Present | 15 (7.5) |
| Yes | 60 (30) | **PNI** | |
| Not available | 67 (33.5) | Not seen | 179 (89.5) |
| **Location** | | Present | 21 (10.5) |
| Tongue | 95 (47.5) | **Margin status** | |
| Floor of mouth | 36 (18) | Free | 134 (67) |
| Hard palate | 8 (4) | Close(<0.1cm) | 32 (16) |
| Buccal mucosa | 22 (11) | Not free | 34 (17) |
| Retromolar area | 7 (3.5) | **Treatment** | |
| Gum | 22 (11) | Surgery alone | 67 (33.5%) |
| Alveolar ridge | 3 (1.5) | Surgery with RT | 100 (50%) |
| **Stage** | | Surgery with RT& | 33 (16.5%) |
| I | 50 (25) | **Recurrence** | |
| II | 38 (19) | No | 170 (85) |
| III | 32 (16) | Yes | 30 (15) |
| IVA | 80 **(**40**)** | | |

*Abbreviation: LVSI, lymphovasular invasion; PNI,perineural invasion RT, radiotherapy; CMT. Chemotherapy

### 3.2. E-cadherin and vimentin expression

Though we constructed TMA using two cores from each case, 10 cases had only single core on slides, as cores had gone missing during processing. Four cases had inadequate tumor in one of cores.

All sections were examined by two observers (Senior pathologist and a third-year resident) separately without knowledge of the clinical data and outcome. Staining intensity and the proportion of reactive tumors cells were assessed. The agreement for intensity between two observers was moderate for both E-cadherin and vimentin, E-cadherin had Kappa 0.475 and 0.443 for first and second core respectively while vimentin had kappa 0.476 and 0.444 for first and second core respectively. The agreement for proportion of reactive tumor cell for E-cadherin was moderated with correlation coefficient of 0.576 and 0.597 for first and second core respectively and strong for vimentin with correlation coefficient of 0.893 and 0.877 for first and second core respectively. We then jointly reassessed all the cores with multi-head microscope and final score was given. The agreement between two cores were moderate, with kappa of 0.41 and 0.494 for E-cadherin and vimentin intensity respectively, the correlation coefficient for proportion of reactive tumor cell was 0.454 and 0.667 for E-cadherin and vimentin respectively.

E-cadherin was mainly localized in the cell membrane while vimentin was localized in cytoplasm. Twenty-eight (14%) tumor showed loss of E- cadherin expression and 172 (86%) showed preserved E-cadherin expression. Vimentin was negative in 113 (56.5%) tumors and positive in 87(43.5%) (Table 4). Figure 4 shows the pictures representative of this classification.

Tumors that had loss of E-cadherin expression showed vimentin expression predominately at stroma tumor interface as shown in Figure 5. E-cadherin and vimentin was negatively correlated with Spearman's rank correlation score of - 0.2, though the correlation was weak it was significant with p-value of 0.001. Figure 6 shows the scatter plot for association between E-cadherin and vimentin.

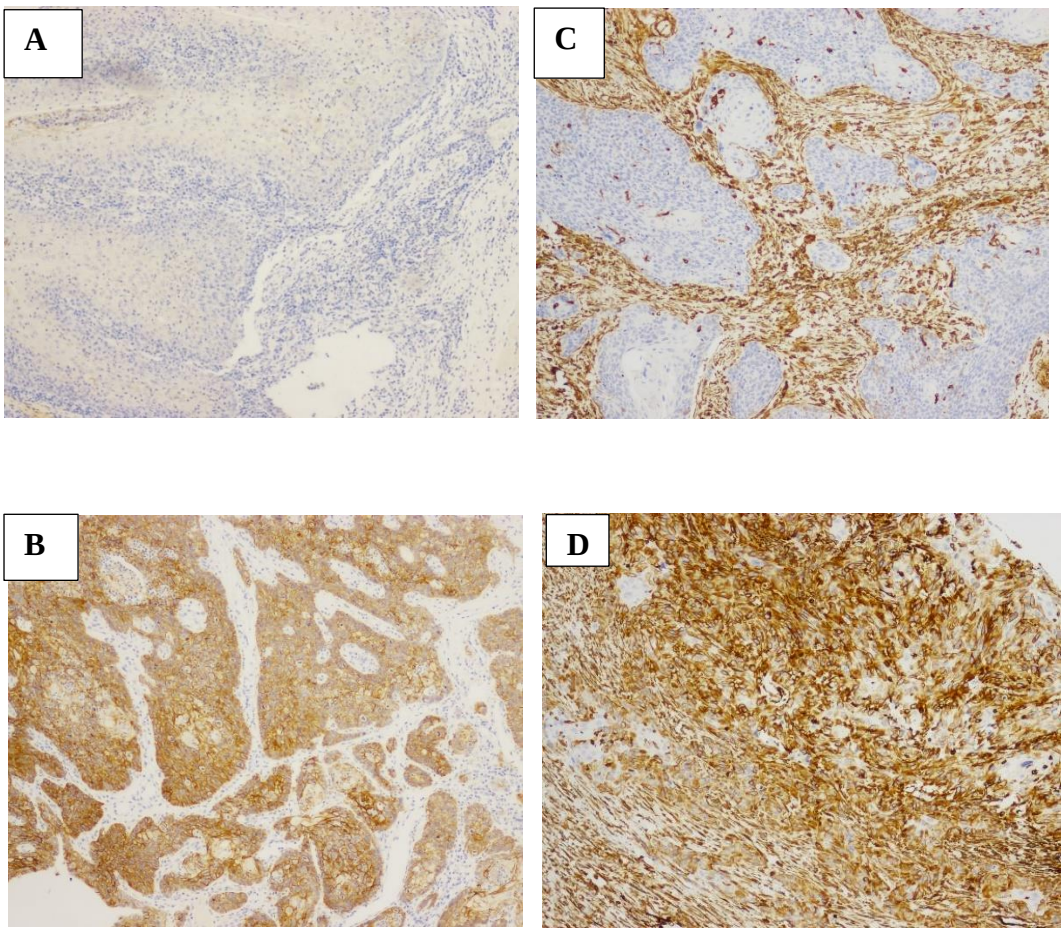


**Figure 4:Immunohistochemical staining of E-cadherin and vimentin in OSCC** at 100× magnification. Tumor with loss of E-cadherin expression *(*A*)*, preserved E-cadherin expression *(*B*)*. Tumor with negative vimentin expression *(*C*)*, and positive vimentin expression *(*D*)*.

**Table 4: E cadherin and vimentin expression**

| E-cadherin expression | Vimentin | | Total (column %) |
|---|---|---|---|
| | Negative | Positive | |
| Loss | 14 | 14 | 28 **(**14%**)** |
| Preserved | 99 | 73 | 172 (86%) |
| Total (Row %) | 113 (56.5%) | 87 (43.5%) | 200 **(**100**)** |

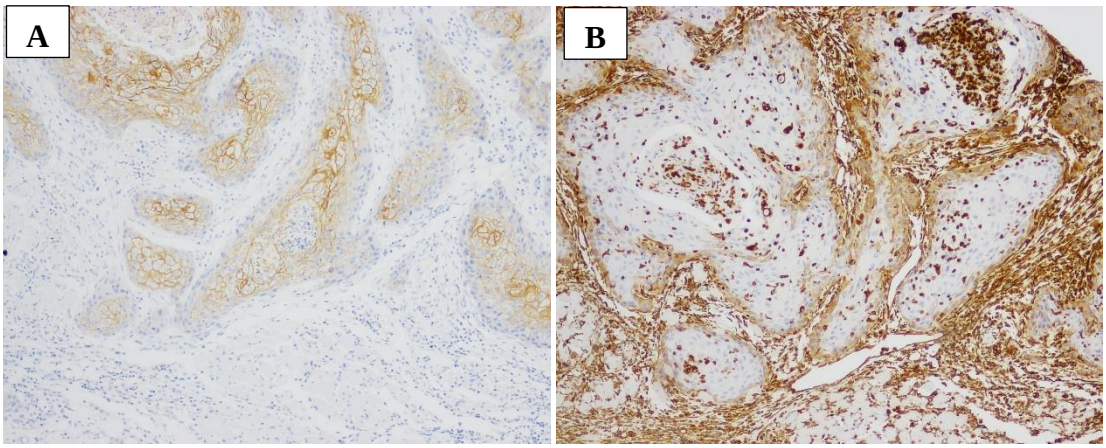


**Figure 5: Staining location of E-cadherin and vimentin**. E-cadherin was expressed mainly at the tumor center *(*A*)* and vimentin was expression mainly at periphery *(*B*)*

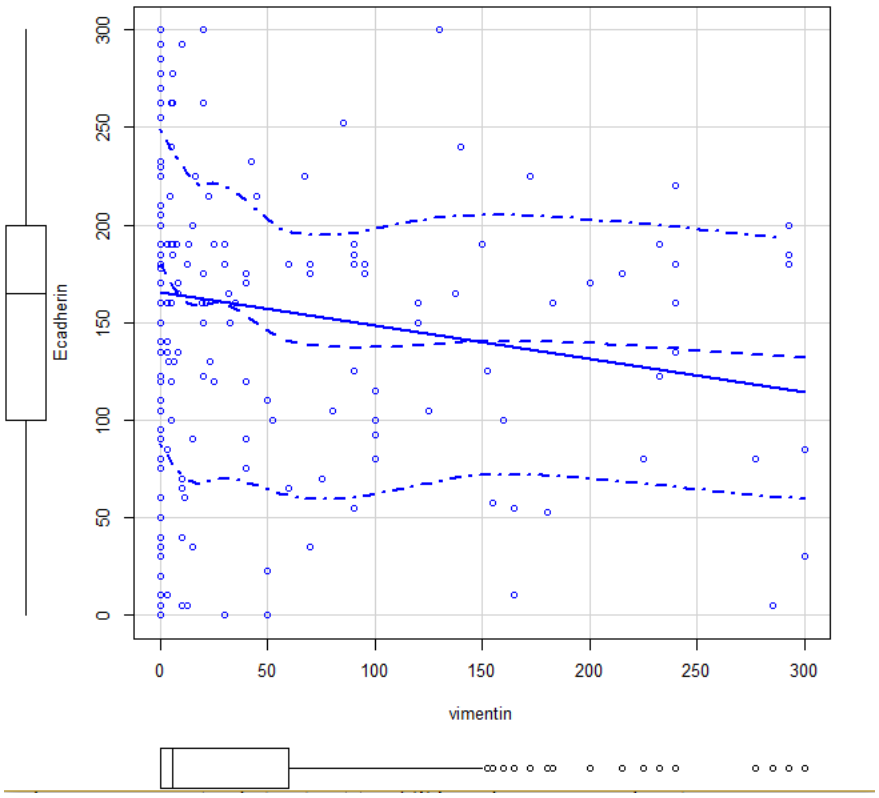


**Figure 6: Scatter plot for E-cadherin and vimentin**.

### 3.3. Relationship of E-cadherin and vimentin expression with clinicopathological features

Table 5 and Table 6 summarizes the relationship of E-cadherin and vimentin with clinicopathological features respectively. E cadherin was significantly associated with lymph node metastasis. Tumors with preserved E-cadherin had higher proportion (67.4%) cases without lymph node metastasis compared and loss of E-cadherin expression.

Though statistically not significant loss of E-cadherin had higher proportion of stage IV (37.5%) tumors and poorly differentiated tumors (7.1%) compared to preserved E-cadherin (57.1% and 2.1% respectively)

Vimentin was not statistically associated with any of the clinicopathological variable. However, tumors with positive vimentin expression had higher proportion of stage IV (43.7%) tumors, poorly differentiated tumor (5.7%) and more lymph node metastasis (25.3%) compared negative vimentin expression (37.2%, 0.9% and 15% respectively).

**Table 5: Association of E-cadherin with clinicopathological variables**

| Variables | Preserved E-cadherin | Loss of E-cadherin | P value |
|---|---|---|---|
| **Total** | 172 | 28 | |
| **Age** | | | 0.211 |
| <=65 | 105 (61) | 13 (46.4) | |
| >65 | 67 (39) | 15 (53.6) | |
| **Gender** | | | 1 |
| Female | 63 (36.6) | 10 (35.7) | |
| Male | 109 (63.4) | 18 (64.3) | |
| **Differentiation** | | | 0.273 |
| Well | 39 (22.7) | 8 (28.6) | |
| Moderate | 129 (75) | 18 (64.3) | |
| Poor | 4 (2.3) | 2 (7.1) | |
| **Stage** | | | 0.16 |
| I | 43 (25) | 7 (25) | |
| II | 35 (20.3) | 3 (10.7) | |
| III | 30 (17.4) | 2 (7.1) | |
| IVA | 64 (37.2) | 16 (57.1) | |
| **T stage** | | | 0.455 |
| T1 | 55 (32) | 10 (35.7) | |
| T2 | 58 (33.7) | 6 (21.4) | |
| T3 | 17 (9.9) | 2 (7.1) | |
| T4 | 42 (24.4) | 10 (35.7) | |
| **N stage** | | | **0.008** |
| N0 | 116 (67.4) | 16 (57.1) | |
| N1 | 28 (16.3) | 1 (3.6) | |
| N2 | 28 (16.3) | 11 (39.3) | |
| **LVSI** | | | 1 |
| Not seen | 159 (92.4) | 26 (92.9) | |
| Present | 13 (7.6) | 2 (7.1) | |
| **Perineural invasion** | | | 0.184 |
| Not seen | 156 (90.7) | 23 (82.1) | |
| Present | 16 (9.3) | 5 (17.9) | |
| **Margin** | | | 0.401 |
| Free | 117 (68) | 17 (60.7) | |
| Close<0.1cm | 25 (14.5) | 7 (25) | |
| Not free | 30 (17.4) | 4 (14.3) | |
| **Treatment** | | | 0.603 |
| Surgery alone | 58 (33.7) | 9 (32.1) | |
| Surgery with RT | 84 (48.8) | 16 (57.1) | |
| Surgery with RT&CMT | 30 (17.4) | 3 (10.7) | |
| **Recurrence** | | | 0.265 |
| No | 144 (83.7) | 26 (92.9) | |
| Yes | 28 (16.3) | 2 (7.1) | |

*Abbreviation: LVSI, lymphovasular invasion; PNI,perineural invasion RT, radiotherapy; CMT. Chemotherapy

**Table 6: Association of vimentin with clinicopathological variables**

| Variables | Negative vimentin | Positive vimentin | P value |
|---|---|---|---|
| **Total** | 113 | 87 | |
| **Age** | | | 0.412 |
| <=65 | 70 (61.9) | 48 (55.2) | |
| >65 | 43 (38.1) | 39 (44.8) | |
| **Gender** | | | 0.056 |
| Female | 34 (30.1) | 39 (44.8) | |
| Male | 79 (69.9) | 48 (55.2) | |
| **Differentiation** | | | 0.167 |
| Well | 27 (23.9) | 20 (23) | |
| Moderate | 85 (75.2) | 62 (71.3) | |
| Poor | 1 (0.9) | 5 (5.7) | |
| **Stage** | | | 0.674 |
| I | 31 (27.4) | 19 (21.8) | |
| II | 23 (20.4) | 15 (17.2) | |
| III | 17 (15) | 15 (17.2) | |
| IVA | 42 (37.2) | 38 (43.7) | |
| **T stage** | | | 0.201 |
| T1 | 41 (36.3) | 24 (27.6) | |
| T2 | 34 (30.1) | 30 (34.5) | |
| T3 | 7 (6.2) | 12 (13.8) | |
| T4 | 31 (27.4) | 21 (24.1) | |
| **N stage** | | | 0.185 |
| N0 | 78 (69) | 54 (62.1) | |
| N1 | 18 (15.9) | 11 (12.6) | |
| N2 | 17 (15) | 22 (25.3) | |
| **LVSI** | | | 0.285 |
| Not seen | 107 (94.7) | 78 (89.7) | |
| Present | 6 (5.3) | 9 (10.3) | |
| **Perineural invasion** | | | 1 |
| Not seen | 101 (89.4) | 78 (89.7) | |
| Present | 12 (10.6) | 9 (10.3) | |
| **Margin** | | | 0.873 |
| Free | 74 (65.5) | 60 (69) | |
| Close<0.1cm | 19 (16.8) | 13 (14.9) | |
| Not free | 20 (17.7) | 14 (16.1) | |
| **Treatment** | | | 0.636 |
| Surgery alone | 41 (36.3) | 26 (29.9) | |
| Surgery with RT | 54 (47.8) | 46 (52.9) | |
| Surgery with RT&CMT | 18 (15.9) | 15 (17.2) | |
| **Recurrence** | | | 0.536 |
| No | 94 (83.2) | 76 (87.4) | |
| Yes | 19 (16.8) | 11 (12.6) | |

*Abbreviation: LVSI, lymphovasular invasion; PNI,perineural invasion RT, radiotherapy; CMT. Chemotherapy

### 3.4. EMT phenotype

EMT is a process where epithelial cells to convert to mesenchymal cells. It is considered completed EMT if there is complete loss of epithelial traits and complete gain of mesenchymal traits and partial EMT without complete loss of epithelial traits or with incomplete gain of mesenchymal traits. To evaluate EMT status we combined E cadherin and vimentin scores. During EMT there is loss of E-cadherin expression and gain of vimentin expression, so we gave reverse score as follows: score 0 for preserved E-cadherin; score 1 for loss of E-cadherin and score 1 for positive vimentin and 0 for negative vimentin.

We then summed the two score. The score ranged from 0-2. Score 0 was considered absent EMT, score 1 as partial EMT and score 2 as complete EMT (Table 7).

Nighty nine (49.5%) tumors showed no EMT, 87(43.5%) show partial EMT and 14(7.5%) had complete EMT phenotype.

**Table 7: EMT categorization**

| Combined Score | Details | EMT | No. (%) |
|---|---|---|---|
| 0 | Preserved E-cadherin + Negative Vimentin | Absent | 99 (49.5%) |
| 1 | Preserved E-cadherin + Positive vimentin or loss of E-cadherin + negative vimentin | Partial | 87 (43.5%) |
| 2 | Loss of E-cadherin + Positive vimentin | Complete | 14 (7%) |

### 3.5. EMT status and association with clinicopathological factors

Table 8 summarizes the association of EMT status with clinicopathological factors. EMT status was significant associated with lymph node metastasis. Tumors with complete EMT had higher proportion (50%) of lymph node metastasis compared to partial EMT (21.8%) and absent EMT (13.1%). Though statically not significant tumors with compete EMT had higher proportions of stage IV tumors and poorly differentiated tumors compared to partial and absent EMT.

**Table 8: Association of EMT status with clinicopathological factors**.

| Variables | Absent EMT | Partial | Complete | P value |
|---|---|---|---|---|
| **Total** | 99 | 87 | 14 | |
| **Age** | | | | 0.154 |
| <=65 | 65 (65.7) | 45 (51.7) | 8 (57.1) | |
| >65 | 34 (34.3) | 42 (48.3) | 6 (42.9) | |
| **Gender** | | | | 0.09 |
| Female | 29 (29.3) | 39 (44.8) | 5 (35.7) | |
| Male | 70 (70.7) | 48 (55.2) | 9 (64.3) | |
| **Differentiation** | | | | 0.067 |
| Well | 24 (24.2) | 18 (20.7) | 5 (35.7) | |
| Moderate | 74 (74.7) | 66 (75.9) | 7 (50) | |
| Poor | 1 (1) | 3 (3.4) | 2 (14.3) | |
| **Stage** | | | | 0.344 |
| I | 27 (27.3) | 20 (23) | 3 (21.4) | |
| II | 22 (22.2) | 14 (16.1) | 2 (14.3) | |
| III | 15 (15.2) | 17 (19.5) | 0 (0) | |
| IVA | 35 (35.4) | 36 (41.4) | 9 (64.3) | |
| **T stage** | | | | 0.71 |
| T1 | 35 (35.4) | 26 (29.9) | 4 (28.6) | |
| T2 | 33 (33.3) | 26 (29.9) | 5 (35.7) | |
| T3 | 6 (6.1) | 12 (13.8) | 1 (7.1) | |
| T4 | 25 (25.3) | 23 (26.4) | 4 (28.6) | |
| **N stage** | | | | **0.024** |
| N0 | 69 (69.7) | 56 (64.4) | 7 (50) | |
| N1 | 17 (17.2) | 12 (13.8) | 0 (0) | |
| N2 | 13 (13.1) | 19 (21.8) | 7 (50) | |
| **LVSI** | | | | 0.532 |
| Not seen | 93 (93.9) | 80 (92) | 12 (85.7) | |
| Present | 6 (6.1) | 7 (8) | 2 (14.3) | |
| **PNI** | | | | 0.773 |
| Not seen | 90 (90.9) | 77 (88.5) | 12 (85.7) | |
| Present | 9 (9.1) | 10 (11.5) | 2 (14.3) | |
| **Margin** | | | | 0.969 |
| Free | 66 (66.7) | 59 (67.8) | 9 (64.3) | |
| Close(<0.1cm) | 15 (15.2) | 14 (16.1) | 3 (21.4) | |
| Not free | 18 (18.2) | 14 (16.1) | 2 (14.3) | |
| **Treatment** | | | | 0.563 |
| Surgery alone | 34 (34.3) | 31 (35.6) | 2 (14.3) | |
| Surgery with RT | 48 (48.5) | 42 (48.3) | 10 (71.4) | |
| Surgery with RT & CMT | 17 (17.2) | 14 (16.1) | 2 (14.3) | |
| **Recurrence** | | | | 0.242 |
| Yes | 82 (82.8) | 74 (85.1) | 14 (100) | |
| No | 17 (17.2) | 13 (14.9) | 0 (0) | |

*Abbreviation: LVSI, lymphovasular invasion; PNI,perineural invasion RT, radiotherapy; CMT. Chemotherapy

### 3.6. Relationship of E-cadherin and vimentin expression with outcome

The mean and median survival of the whole group were 37 months and 48 months, respectively. E-cadherin (0.006), vimentin (P = 0.001) and EMT status (P < 0.001) were all associated with 5-year survival outcome in Kaplan Meier analysis. The Kaplan Meier graph for survival analysis are shown in Figure 7,8 and 9 respectively. As age and treatment were the other significant variable, we have plotted Kaplan Meier graph as shown in Figure 10 and 11 respectively.

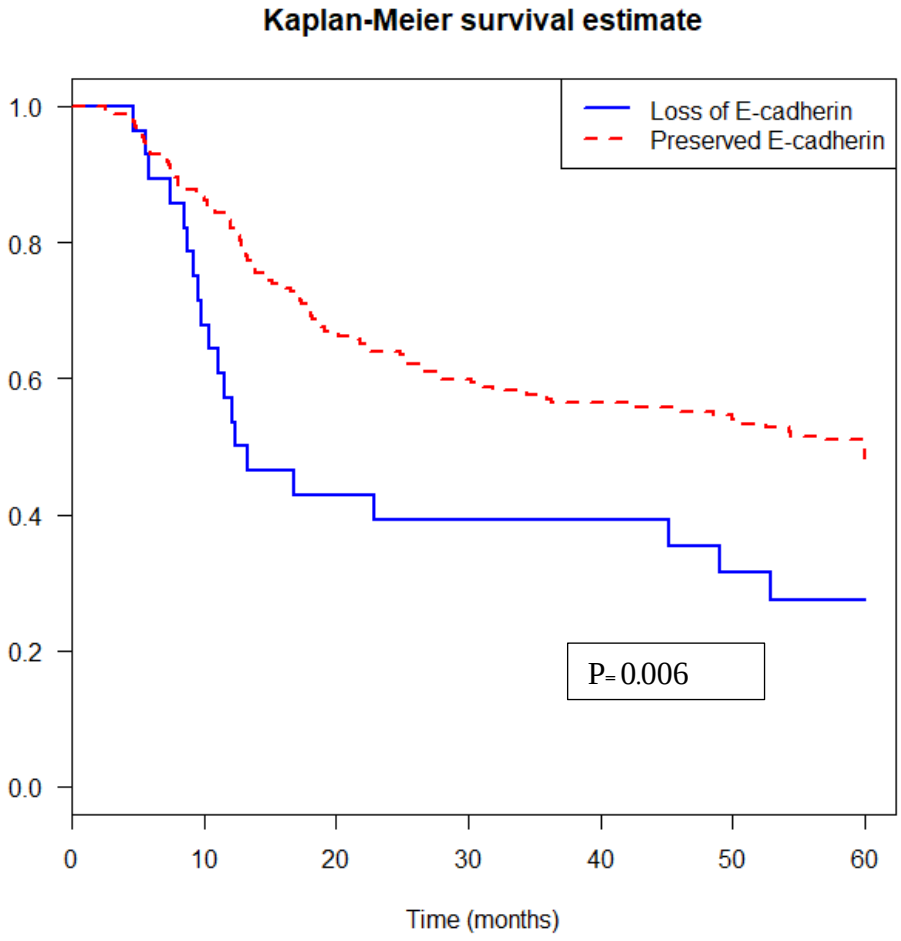


**Figure 7: Kaplan Meire overall survival graph for E-cadherin**

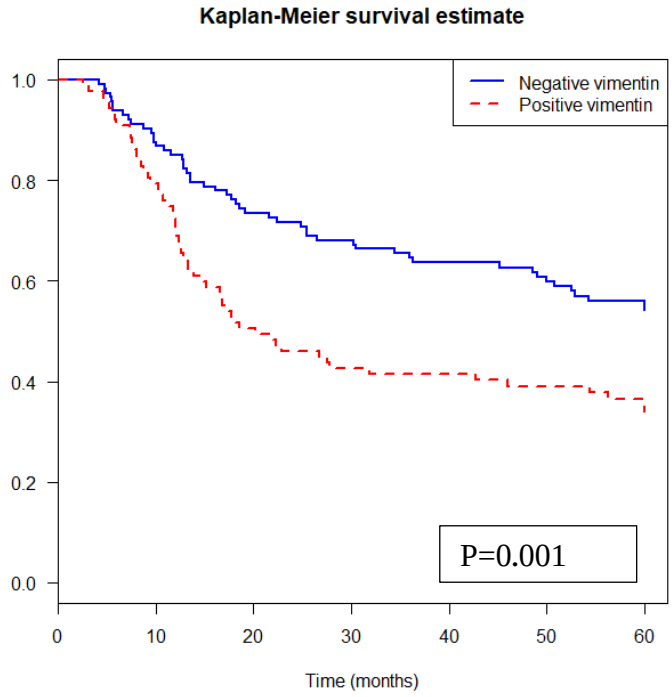


**Figure 8: Kaplan Meier overall survival graph for vimentin**

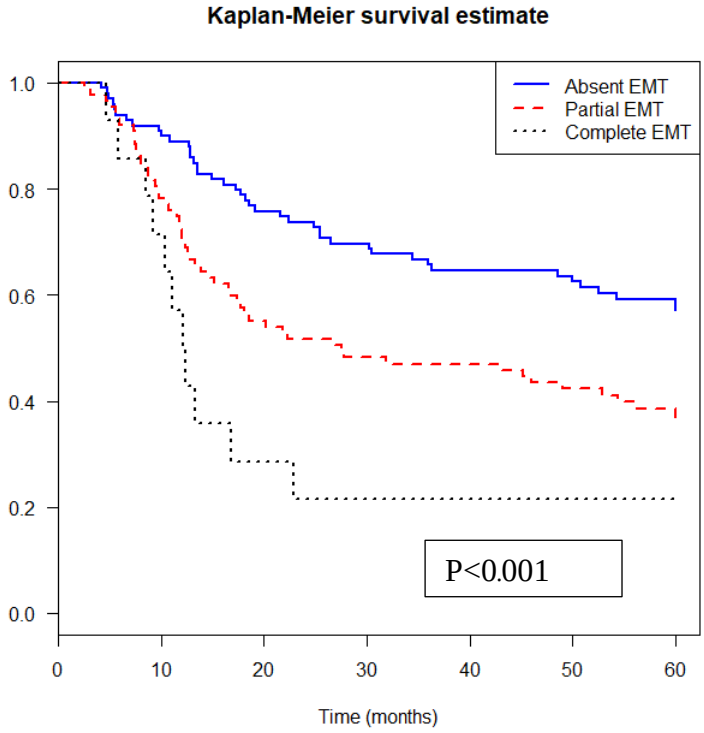


**Figure 9: Kaplan Meier overall survival graph for EMT status**

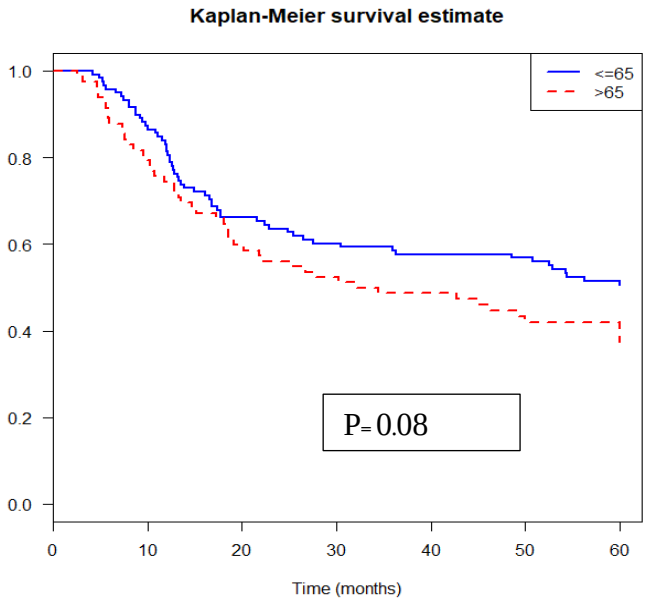


**Figure 10: Kaplan Meier survival curve for Age**

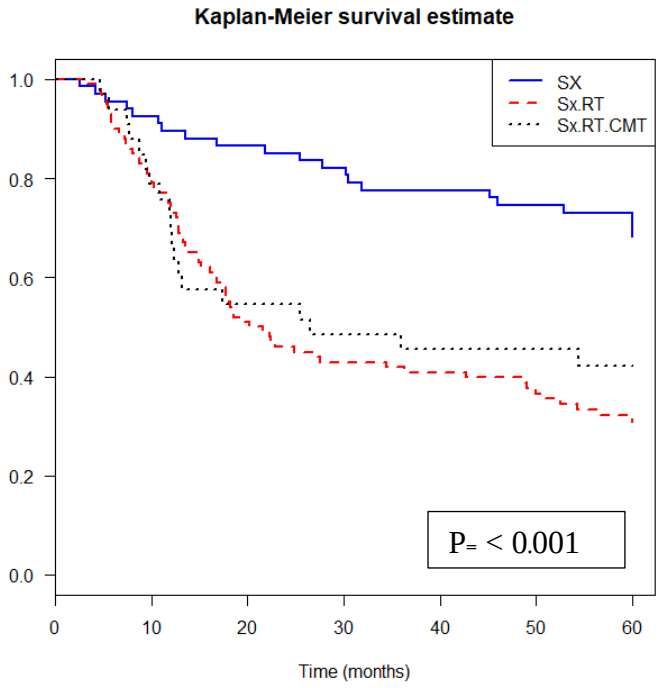


**Figure 11: Kaplan Meier survival curve for treatment**

In univariate cox analysis, both E-cadherin and vimentin were significantly associated with 5-year overall survival outcome (Table 9), with HR 1.94, 95%CI (1.19-3.16) for loss of E-cadherin expression and HR 1.85, 95%CI (1.26-2.7) for positive vimentin expression. For the EMT status, as the EMT progressed the HR increased with partial EMT having HR of 1.88, 95%CI (1.26-2.81) and complete EMT having HR of 3.33, 95%CI (1.71-6.51). The other variables that were significantly associated with outcome in univariate analysis were stage (HR 4.48, 95% CI (2.54-7.91)), T size stage (HR 4.09, 95% CI (2.41-6.95)), N node staging (HR 2.32, 95% CI (1.48-3.66)), recurrence (HR 0.56, 95% CI (0.31-1.02)) and treatment (HR 3.15,95%CI (1.92-5.15)).

**Table 9: Univariate Cox regression analysis of overall survival**

| Variables | HR | 95% CI | P Wald | P. LR |
|---|---|---|---|---|
| **Age** | | | | 0.081 |
| <=65 | 1 | | | |
| >=65 | 1.4 | 0.96-2.05 | 0.079 | |
| **Gender** | | | | 0.093 |
| Male | 1 | | | |
| Female | 0.72 | 0.49-1.05 | 0.089 | |
| **Location** | | | | 0.127 |
| Tongue | 1 | | | |
| Floor of mouth | 1.28 | 0.76-2.16 | 0.355 | |
| Hard palate | 2.47 | 1.05-5.81 | 0.038 | |
| Lip | 0.78 | 0.24-2.52 | 0.679 | |
| Buccal/ cheek mucosa | 1.46 | 0.79-2.72 | 0.228 | |
| Retromolar area | 2.78 | 1.1-7.02 | 0.031 | |
| Gum | 2.06 | 1.15-3.72 | 0.016 | |
| Alveolar ridge | 1.22 | 0.3-5.05 | 0.78 | |
| **Differentiation** | | | | 0.671 |
| Moderate | 1 | | | |
| Well | 0.83 | 0.54-1.3 | 0.422 | |
| Poor | 0.69 | 0.21-2.27 | 0.539 | |
| **Stage** | | | | **< 0.001** |
| I | | | | |
| II | 1.54 | 0.76-3.11 | 0.23 | |
| III | 2.17 | 1.07-4.4 | 0.031 | |
| IV | 4.48 | 2.54-7.91 | < 0.001 | |
| **T stage** | | | | **< 0.001** |
| T1 | 1 | | | |
| T2 | 1.7 | 0.97-2.95 | 0.062 | |
| T3 | 3.84 | 1.95-7.58 | < 0.001 | |

| | | | | |
|---|---|---|---|---|
| T4 | 4.09 | 2.41-6.95 | < 0.001 | |
| **N stage** | | | | **0.002** |
| N0 | 1 | | | |
| N1 | 1.64 | 0.97-2.77 | 0.064 | |
| N2 | 2.32 | 1.48-3.66 | < 0.001 | |
| **LVSI** | | | | 0.102 |
| Absent | 1 | | | |
| Present | 1.75 | 0.94-3.27 | 0.079 | |
| **PNI** | | | | 0.53 |
| Absent | 1 | | | |
| Present | 1.22 | 0.67-2.22 | 0.519 | |
| **Margin** | | | | 0.141 |
| Free | 1 | | | |
| Free but close(<0.1cm) | 0.69 | 0.38-1.25 | 0.223 | |
| Not free | 1.38 | 0.85-2.24 | 0.195 | |
| **Treatment** | | | | **< 0.001** |
| Surgery alone | 1 | | | |
| Surgery with RT | 3.15 | 1.92-5.15 | < 0.001 | |
| Surgery with RT& CMT | 2.53 | 1.36-4.71 | 0.003 | |
| **Recurrence** | | | | **0.043** |
| Yes | 1 | | | |
| No | 0.56 | 0.31-1.02 | 0.06 | |
| **E-cadherin** | | | | **0.013** |
| Preserved | 1 | | | |
| Loss | 1.94 | 1.19-3.16 | 0.008 | |
| **Vimentin** | | | | **0.002** |
| Negative | 1 | | | |
| Positive | 1.85 | 1.26-2.7 | 0.002 | |
| **EMT** | | | | |
| Absent | 1 | | | **<0.001** |
| Partial | 1.88 | 1.26-2.81 | 0.002 | |
| Complete | 3.33 | 1.71-6.51 | < 0.001 | |

*Abbreviation: LVSI, lymphovasular invasion; PNI,perineural invasion RT, radiotherapy; CMT. Chemotherapy

Table 10 shows the results of multivariate cox regression analysis for overall survival. Both E-cadherin and vimentin were scientifically associated with outcome with HR 1.74, 95%CI (1.04-2.93) and HR1.64, 95%CI (1.12-2.41) respectively. The other significant prognostic factors were age, stage and treatment. Patients who had received adjunct radiotherapy and chemotherapy had higher hazard ratio compared to those who had only surgical treatment.

**Table 10: Multivariate Cox's regression analysis for overall survival with individual protein**

| Variables | crude HR 95%CI | adj. HR 95%CI | P Wald test | P LR test |
|---|---|---|---|---|
| **Age** | | | | 0.002 |
| <=65 | 1 | 1 | | |
| >65 | 1.4 (0.96-2.05) | 1.94 (1.28-2.95) | 0.002 | |
| **Stage** | | | | < 0.001 |
| I | 1 | 1 | | |
| II | 1.54 (0.76-3.11) | 1.38 (0.67-2.84) | 0.377 | |
| III | 2.17 (1.07-4.4) | 2.21 (1.07-4.57) | 0.032 | |
| IV | 4.48 (2.54-7.91) | 3.41 (1.86-6.25) | < 0.001 | |
| **Treatment** | | | | 0.005 |
| Surgery alone | 1 | 1 | | |
| Surgery with RT | 3.15 (1.92-5.15) | 2.27 (1.34-3.86) | 0.002 | |
| Surgery, RT& CMT | 2.53 (1.36-4.71) | 2.33 (1.16-4.69) | 0.017 | |
| **E-cadherin** | | | | 0.045 |
| Preserved | 1 | 1 | | |
| Loss | 1.94 (1.19-3.16) | 1.74 (1.04-2.93) | 0.036 | |
| **Vimentin** | | | | 0.011 |
| Negative | 1 | 1 | | |
| Positive | 1.85 (1.26-2.7) | 1.64 (1.12-2.41) | 0.011 | |

*Abbreviation:RT, radiotherapy; CMT. Chemotherapy

When EMT status was analyzed in multivariate cox regression, it showed higher hazard ratio compared to individual proteins (Table 11)**.** The hazard ratio increased with progression of EMT score, with HR of 1.64, 95%CI (1.09-2.49) for partial EMT and HR 2.88, 95%CI (1.44-5.79) for complete EMT**.** The other significant variables were same as multivariate cox analysis with individual protein**.**

**Table 11: Multivariate Cox s regression analysis for overall survival with EMT status**

| **Variables** | **Crude HR 95%CI.** | **adj. HR 95%CI** | **P Walds test** | **P LR test** |
|---|---|---|---|---|
| **Age** | | | | 0.002 |
| <=65 | 1 | 1 | | |
| >65 | 1.4 (0.96-2.05) | 1.95 (1.29-2.97) | 0.002 | |
| **Stage** | | | | < 0.001 |
| I | 1 | 1 | | |
| II | 1.54 (0.76-3.11) | 1.38 (0.67-2.83) | 0.38 | |
| III | 2.17 (1.07-4.4) | 2.21 (1.07-4.59) | 0.033 | |
| IV | 4.48 (2.54-7.91) | 3.43 (1.86-6.33) | < 0.001 | |
| **Treatment** | | | | 0.006 |
| Surgery alone | 1 | 1 | | |
| Surgery with RT | 3.15 (1.92-5.15) | 2.26 (1.32-3.86) | 0.003 | |
| Surgery, RT& CMT | 2.53 (1.36-4.71) | 2.32 (1.16-4.64) | 0.018 | |
| **EMT** | | | | 0.006 |
| Absent | 1 | 1 | | |
| Partial | 1.88 (1.26-2.81) | 1.64 (1.09-2.49) | 0.019 | |
| Complete | 3.33 (1.71-6.51) | 2.88 (1.44-5.79) | 0.003 | |

*Abbreviation:RT, radiotherapy; CMT. Chemotherapy

# Chapter 4

# Discussion

Our study used immunohistochemical technique on paraffin-embedded microarray tissue slides, to evaluate expression of E-cadherin and vimentin individually and combined (EMT status) and to investigate the association of their expression with clinicopathological factors and overall 5 years survival in OSCC.

In carcinogenesis EMT confers an invasive phenotype to cancer cell and act as a key regulator of metastasis and invasion (8). Additionally, EMT confers cancer cells stem cell properties (76) and has been reported to be responsible for resistance to chemotherapy and immunotherapy (11). A vast range of proteins play role in EMT of which loss of expression of E cadherin and high vimentin expression are considered as EMT markers in HNSCC (47, 48).

E-cadherin is a vital molecule responsible for cell to cell adhesion and loss of E-cadherin increases the mobility of epithelial cells subsequently leading to local infiltration (77). In our study E-cadherin was significantly associated with lymph node metastasis (P=0.008). Tumors with loss of E-cadherin expression had more lymph node metastasis. Zhou et al. (67)and Pyo et al. (13) also reported that reduced expression of E-cadherin to be associated with lymph node metastasis. Study of Huber et al. (78) showed that down regulation of E-cadherin could even predict occult metastasis in lymph node biopsy suggesting that E-cadherin could help to plan the extent of surgery.

E-cadherin was significantly associated with 5 years overall survival in OSCC in univariate analysis (HR 1.94, 95%CI (1.19-3.16)) and in multivariate analysis (HR 1.74, 95%CI (1.04-2.93)). Our findings were concordant with Fan et al. (69) who also reported association of E-cadherin expression with prognosis but discordant with. Liu et al (57) who found no association. The discrepancy could be due to difference in grouping of expression. Liu et al had grouped protein expression in three groups. Other than head and neck cancer, reduced E-cadherin expression has been reported to be associated with poor prognosis in other cancers of esophagus (52), stomach (53) and ovary (53).

Vimentin is a mesenchymal protein and expression of vimentin is not seen in normal epithelial cells. Vimentin expression in carcinoma corresponds with a migratory phenotype. Its expression has been associated with increased invasions (56)

Sawant et al reported vimentin expression to be associated with clinical stage, and regional lymph node metastases in oral cancer. In our study, vimentin was not associated with any other clinicopathological variable. However, though statistically not significant, tumors with strong vimentin expression had higher proportion of stage 4 (57.1%) and N2 nodal metastasis (42.9%).

Vimentin was significant associated to 5-year overall survival in OSCC in univariate analysis (HR 1.85, 95%CI (1.26-2.7)). and multivariate analysis (HR 1.64, 95%CI (1.12-2.41)). Our study is concordant with Liu et al. (12) and Sawant et al. (14) and discordant with Silva et al. (65). The discrepancy with finding of Silva et al. could be due to different inclusion criteria as they had analyzed the protein expression in cases with multiple primary tumors. Other than oral cancer, vimentin expression has been reported to be associated with tumor invasion and a poor prognosis in cancers of breast(58), colorectal carcinoma (59), and lung cancer (60, 79).

We observed that tumors that had loss of E-cadherin expression showed vimentin expression, predominately at stroma tumor interface. This observation was further confirmed by statistically significant negative correlation between which E-cadherin and vimentin. In previous studies on OSCC cell lines, E-cadherin was shown to be downregulated with upregulation of vimentin (79), similar finding was also seen in tumors of breast (80) and intrahepatic cholangiocarcinoma (81).

A combined evaluation was performed using both E-cadherin and vimentin to determine the prognostic significance of EMT status. EMT status was significantly associated with lymph node metastasis (P=0.024). Tumors with complete EMT had higher proportion of lymph node metastasis compared to partial EMT and absent EMT. In univariate analysis, tumors with complete EMT had far more greater hazard ratio 3.33, 95%CI (1.71-6.51) than individual E-cadherin and vimentin (HR 1.94 and HR 1.85 respectively). Multivariate study further confirmed EMT status to be an independent strong prognostic indicator in OSCC. The hazard ratio increased with progression of EMT, with partial EMT having HR 1.64, 95%CI (1.09-2.49) and

complete EMT having HR of 2.88, 95%CI (1.44-5.79), which was the higher compared to individual protein. To the best of our knowledge this is the first study to analyze combined E-cadherin and vimentin expression to evaluate prognostic significance of EMT status in oral cancer. Cunha et al. (82)reported similar finding like our study in penile carcinoma, with complete EMT status having highest hazard ratio (HR 7.637, 95% CI (3.153–18.496)) compared to all the other significant prognostic indicators. They, however, have not analyzed the prognostic significance with E-cadherin and vimentin separately. In another study by Aruga et al.(66), they evaluated EMT phenotype in lung squamous cell carcinoma by combining E-cadherin and vimentin. They found EMT status to be a significant indicator of poor prognosis (HR, 2.695; 95% CI, 1.064-6.82, P = 0.036).

According to the WHO classification of head and neck tumors, the most significant prognostic factors in oral cancer are the tumor size, lymph node metastasis status and distant metastasis. The other significant prognostic factor in our study was age more than 65, stage and treatment. In our study there were no cases of tumor with distant metastasis. Our result show that older people have poorer prognosis which is consistent with study of Pruegsanusa et al. (83), conducted on 410 oral cancer in Songklanagarind hospital. They also reported patients who had both surgery and radiotherapy to had better survival outcome (HR 0.62, 95% CI (0.45-0.84)). Contrary to their report we found patient who had both surgery and adjunct radiotherapy to have poorer survival. The likely cause of this discrepancy is that we had lesser sample size. The other possibility is that adjunct radiotherapy and chemotherapy may lead to poorer prognosis. According to the study conducted in United States where they had included all oral cancer patients in National Cancer Data Base from 1998 to 2011 (21) they found that non-surgical treatment to be associated with decreased overall survival in both early and late stage. More studies are required with larger sample size to truly determine the prognostic significant of adjunct radiotherapy.

As we conducted retrospective study, in some cases information regarding smoking (12%), alcohol (16%) and betel consumption (33.5%) habits were missing. We cannot undermine the effects of these risk factors on our protein expression and outcome. The other possible limitation is that we might have missed cases of tumor

recurrences as the patients might have gone to a different hospital for treatment. We have not investigated disease free survival, so we cannot undermine the effects of recurrence on outcome

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# Chapter 5

## Conclusion

In our study loss of E-cadherin and EMT status was significantly associated with lymph node metastasis. Though both E-cadherin and vimentin expression could act as an independent prognostic factor, a combined evaluation of E-cadherin and vimentin expression to evaluate EMT status could provide a better indicator of prognosis in OSCC in addition to age, stage and treatment modality in surgically resected oral squamous cell carcinoma.

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# Appendix

## 1. Cellular events during EMT

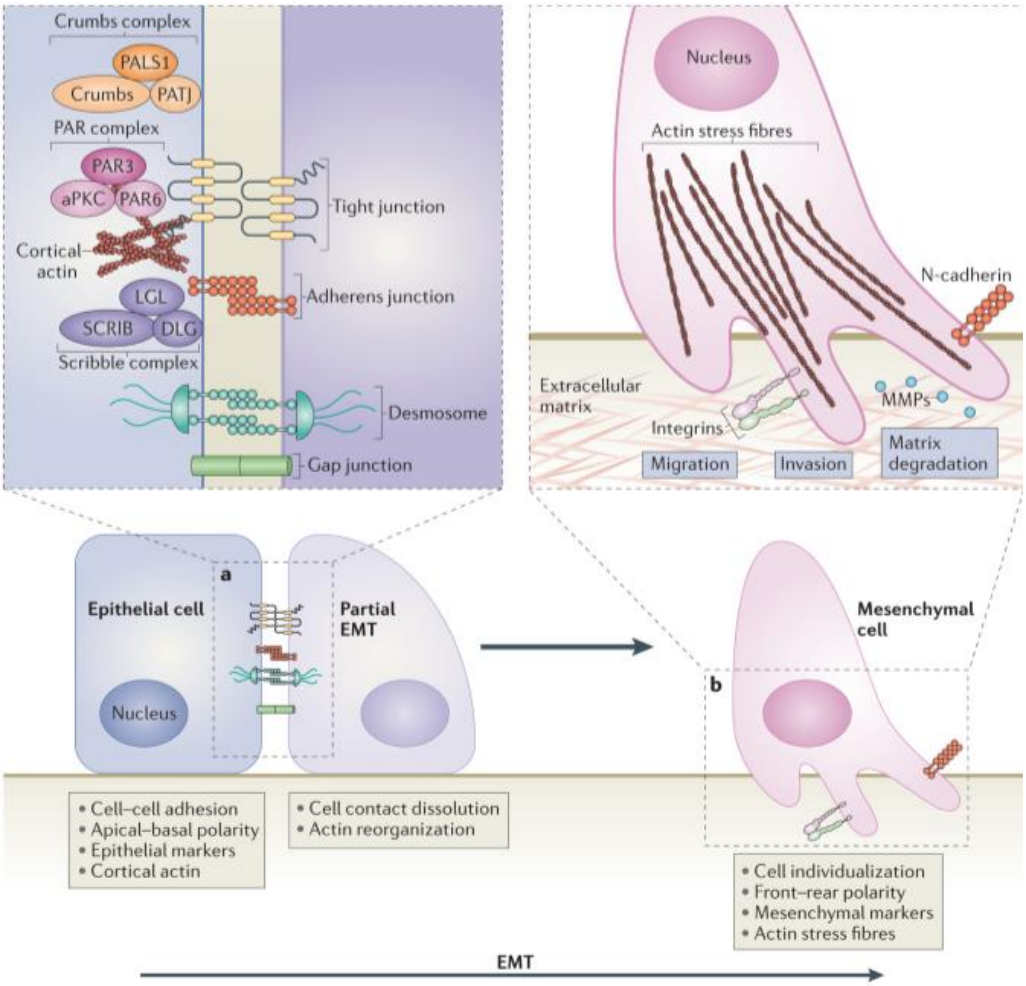


Figure 1. Cellular events during EMT (Lamouille S. et al. Nat Rev Mol Cell Biol. 2014 March; 15(3): 178–196)

a | During epithelial–mesenchymal transition (EMT) firstly there is disassembly of epithelial cell–cell contacts

b | Next, the epithelial actin architecture reorganizes, and cells acquire motility and invasive capacities by expressing matrix metalloproteinases (MMPs) that can degrade extracellular matrix (ECM) proteins

# VITAE

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