STUDY OF THE MORPHOLOGICAL AND FUNCTIONAL ALTERATIONS OF HIGH ENDOTHELIAL VENULES IN THE REGIONAL LYMPH NODES OF TONGUE CANCER PATIENTS AND ITS CLINICO-PATHOLOGICAL CORRELATIONS

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Dr. Lee Ser Yee

2011

Title:

RETROSPECTIVE STUDY OF THE MORPHOLOGICAL AND FUNCTIONAL ALTERATIONS OF HIGH ENDOTHELIAL VENULES IN THE REGIONAL LYMPH NODES OF TONGUE CANCER PATIENTS AND ITS CLINICO-PATHOLOGICAL CORRELATIONS

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1. Summary

Squamous cell carcinoma of the tongue is one of the most prevalent tumors of the head and neck region. The extent of lymph node metastasis is a major determinant for the staging, the most reliable adverse factor for prognosis of squamous cell carcinoma of the tongue and it guides therapeutic decisions. The Paget's "Seed and Soil" theory for cancer and its metastasis is well known and established. Angiogenesis and lymphangiogenesis are both important processes contributing to tumor progression and metastasis. Cancer research has been driven to understand tumor-induced angiogenesis and lymphangiogenesis. Primary tumors can induce lymph channel and vasculature reorganizations within sentinel lymph nodes before the arrival of cancer cells. The key blood vessels in such lymph nodes that are remodeled are identified as high endothelial venules (HEV). The morphological alteration of HEV in the presence of a cancer, coupled with the increased proliferation rate of the endothelial cells, results in a functional shifting of HEV from immune response mediator to blood-flow carrier. Previous studies have demonstrated the role of HEV in inflammatory setting. It was demonstrated that a cancer-induced reorganization is quite different from an endotoxin-induced inflammatory alteration. Our preliminary studies of HEV and its role in lymph nodes of patients with squamous cell carcinoma of the tongue with clinico-pathological correlations revealed a relationship between HEV, cancer metastasis and clinical outcome. These pathological processes are reviewed and clinical phenomena explained in the aid of developing novel therapeutics and prevention strategies against cancer metastasis in the future.

2. List of Tables

Table 1

American Joint Committee on Cancer Staging for Tongue cancer

American Joint Committee on Cancer (AJCC) Staging

- Critical for treatment selection, prognosis and comparison of results.

Cancer stages	
Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor 2 cm or less in greatest dimension
T2	Tumor more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumor more than 4 cm in greatest dimension
T4 (lip)	Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face, i.e., chin or nose
T4a	(oral cavity) Tumor invades adjacent structures (e.g., through cortical bone, into deep [extrinsic] muscle of tongue [genioglossus, hyoglossus, palatoglossus, and styloglossus], maxillary sinus, skin of face)
T4b	Tumor invades masticator space, pterygoid plates, or skull base and/or encases internal carotid artery
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Greene, F. L., Page, D. L., Fleming, I. D., Fritz, A. G., Balch, C.M., Haller, D. G., et al. (2002). AJCC Cancer Staging Manual, 6th edn. New York: Springer.

Table 2 Adverse features of tongue SCC

Adverse features of Tongue Cancer	
1.	Extracapsular nodal spread (ECS)
2.	Positive margins
3.	pT3 or pT4 primary
4.	N2 or N3 nodal disease
5.	Nodal disease in Levels IV or V
6.	Perineural invasion
7.	Vascular embolism

Table 3: Immunohistochemistry Protocol

Steps	Process	Time
1	Deparaffinization	
2	Antigen Retrieval	20 mins
3	Rinse with Phosphate Buffered Saline(PBS) briefly	2 mins
4	Add 3% Hydrogen Peroxide- incubate	15 mins
5	Wash with PBS	5 mins
6	Add Horse Serum (blocking serum)- incubate	15 mins
7	Add Primary antibody (anti-MECA 79), incubate overnight	12 hours
8	Wash with PBS and put on belly dancer	5 mins
9	Add Biotinylated secondary antibody	10 mins
10	Wash with PBS and put on belly dancer	5 mins
11	Add Streptavidin/peroxidase- incubate	5 mins
12	Wash with PBST X 2 times (5 mins each time)	10 mins
13	Wash with Antibody Dilution Buffer (PBE) 3 times (3 mins each time)	9 mins
14	Add Novo Red- develop for 10 mins	10 mins
15	Counter stain IHC	

Table 4 Summary of results

HEV parameters	Clinical data	Relative Risk	p
Total no. of HEVs (A)	Overall Survival	1.024	0.471
	Disease Free Interval	1.051	**0.022**
Total no. of HEVs (A) and Disease Free Interval (as a Cohort)	Disease Free Interval	1.051	**0.023**
Dilated HEVs (B)	Overall Survival	1.071	0.476
	Disease Free Interval	1.034	0.594
HEV with rbcs within its lumen (C)	Overall Survival	1.116	0.345
	Disease Free Interval	1.044	0.584
Ratio of dilated HEVs to the total no. of HEVs(**B**/**A**)	Overall Survival	1.078	0.982
	Disease Free Interval	10.10	0.450
Ratio of dilated HEVs with rbcs to total no. HEV (**C**/**A**)	Overall Survival	3.624	0.737
	Disease Free Interval	4.67	0.643
Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs (**C**/**B**)	Overall Survival	17.884	0.171
	Disease Free Interval	5.458	0.208

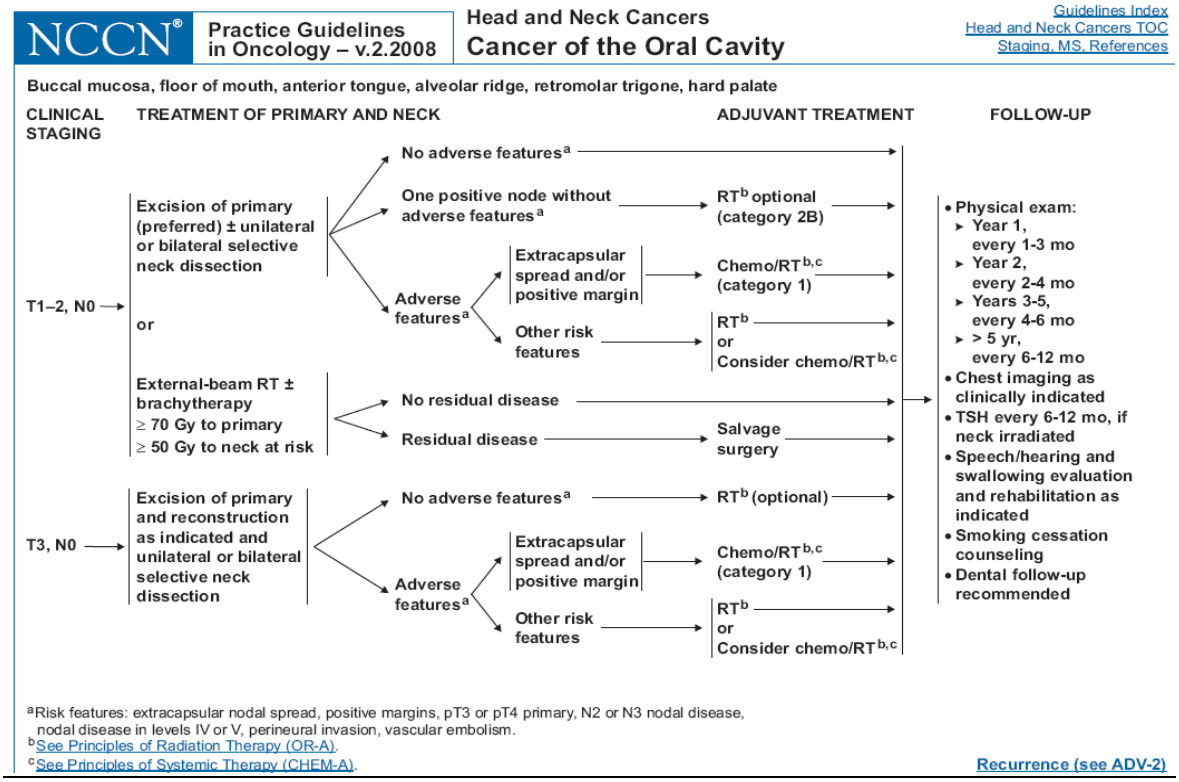
Table 5

Summary of the secondary analysis in the supplementary data

Tumor Characteristics	Clinical data	Relative Risk	p
Tumor volume (TV)	Overall Survival	0.985	0.476
	Disease Free Interval	0.990	0.481
Stage (S)	Overall Survival	1.116	0.71
	Disease Free Interval	1.302	0.348
Grade(G)	Overall Survival	0.882	0.815
	Disease Free Interval	1.436	0.308
Since there are no statistical difference noted between the 2 groups, we now consider the 2 groups (Cases and Controls) as a cohort and repeat the analysis summarized below (i.e. without considering the group)			
Tumor volume (TV_c)	Overall Survival	0.994	0.765
	Disease Free Interval	0.994	0.648
Stage (S_c)	Overall Survival	1.364	0.327
	Disease Free Interval	1.209	0.255
Grade(G_c)	Overall Survival	1.121	0.822
	Disease Free Interval	1.493	0.221

3. List of Figures

Figure 1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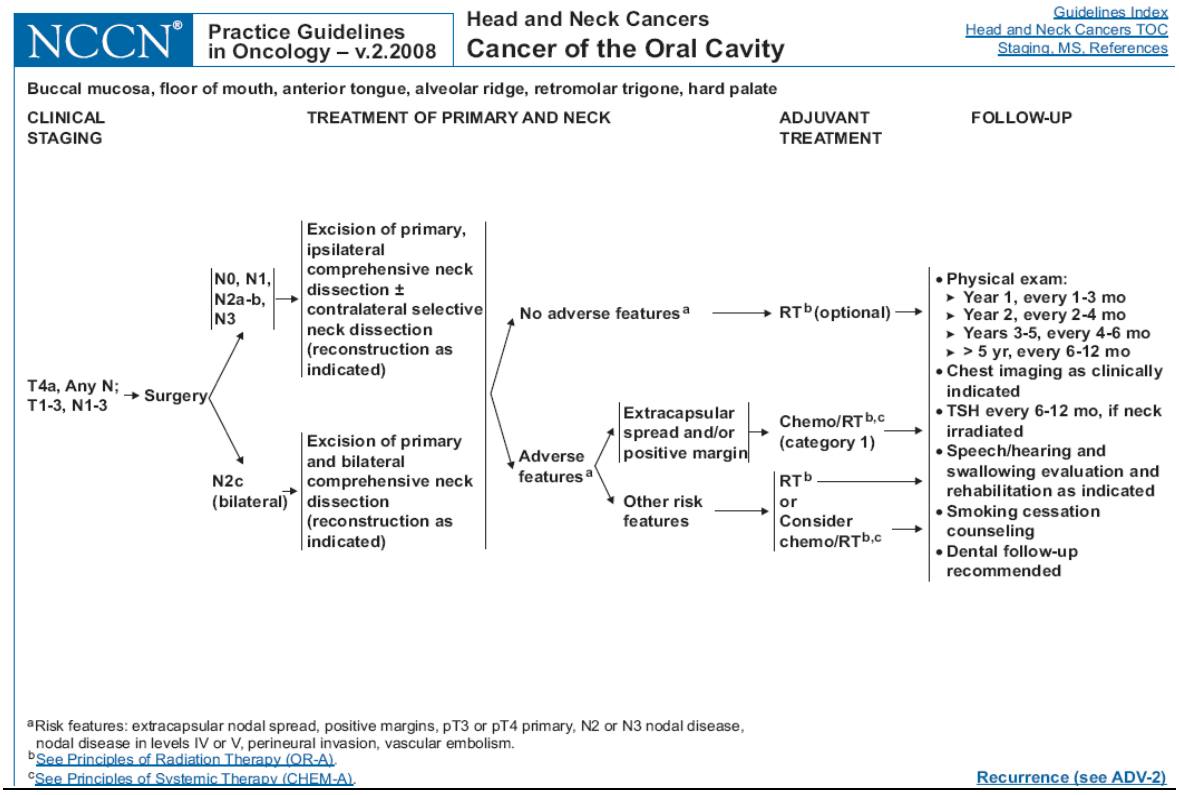
Staging, MS, References
Buccal mucosa, floor of mouth, anterior tongue, alveolar ridge, retromolar trigone, hard palate
CLINICAL STAGING
TREATMENT OF PRIMARY AND NECK
ADJUVANT TREATMENT
FOLLOW-UP
T1–2, N0
Excision of primary (preferred) ± unilateral or bilateral selective neck dissection
or
External-beam RT ± brachytherapy
≥ 70 Gy to primary
≥ 50 Gy to neck at risk
No adverse features[a]
One positive node without adverse features[a]
Adverse features[a]
Extracapsular spread and/or positive margin
Other risk features
No residual disease
Residual disease
RT[b] optional (category 2B)
Chemo/RT[b,c] (category 1)
RT[b] or Consider chemo/RT[b,c]
Salvage surgery
T3, N0
Excision of primary and reconstruction as indicated and unilateral or bilateral selective neck dissection
No adverse features[a]
Adverse features[a]
Extracapsular spread and/or positive margin
Other risk features
RT[b] (optional)
Chemo/RT[b,c] (category 1)
RT[b] or Consider chemo/RT[b,c]
• Physical exam:
▸ Year 1, every 1-3 mo
▸ Year 2, every 2-4 mo
▸ Years 3-5, every 4-6 mo
▸ > 5 yr, every 6-12 mo
• Chest imaging as clinically indicated
• TSH every 6-12 mo, if neck irradiated
• Speech/hearing and swallowing evaluation and rehabilitation as indicated
• Smoking cessation counseling
• Dental follow-up recommended
[a]Risk features: extracapsular nodal spread, positive margins, pT3 or pT4 primary, N2 or N3 nodal disease, nodal disease in levels IV or V, perineural invasion, vascular embolism.
[b]See Principles of Radiation Therapy (OR-A).
[c]See Principles of Systemic Therapy (CHEM-A).
Recurrence (see ADV-2)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CLINICAL STAGING
TREATMENT OF PRIMARY AND NECK
ADJUVANT TREATMENT
FOLLOW-UP
T4a, Any N; T1-3, N1-3
Surgery
N0, N1, N2a-b, N3
N2c (bilateral)
Excision of primary, ipsilateral comprehensive neck dissection ± contralateral selective neck dissection (reconstruction as indicated)
Excision of primary and bilateral comprehensive neck dissection (reconstruction as indicated)
No adverse features[a]
Adverse features[a]
Extracapsular spread and/or positive margin
Other risk features
RT[b] (optional)
Chemo/RT[b,c] (category 1)
RT[b] or Consider chemo/RT[b,c]
• Physical exam:
▸ Year 1, every 1-3 mo
▸ Year 2, every 2-4 mo
▸ Years 3-5, every 4-6 mo
▸ > 5 yr, every 6-12 mo
• Chest imaging as clinically indicated
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[a]Risk features: extracapsular nodal spread, positive margins, pT3 or pT4 primary, N2 or N3 nodal disease, nodal disease in levels IV or V, perineural invasion, vascular embolism.
[b]See Principles of Radiation Therapy (OR-A).
[c]See Principles of Systemic Therapy (CHEM-A).
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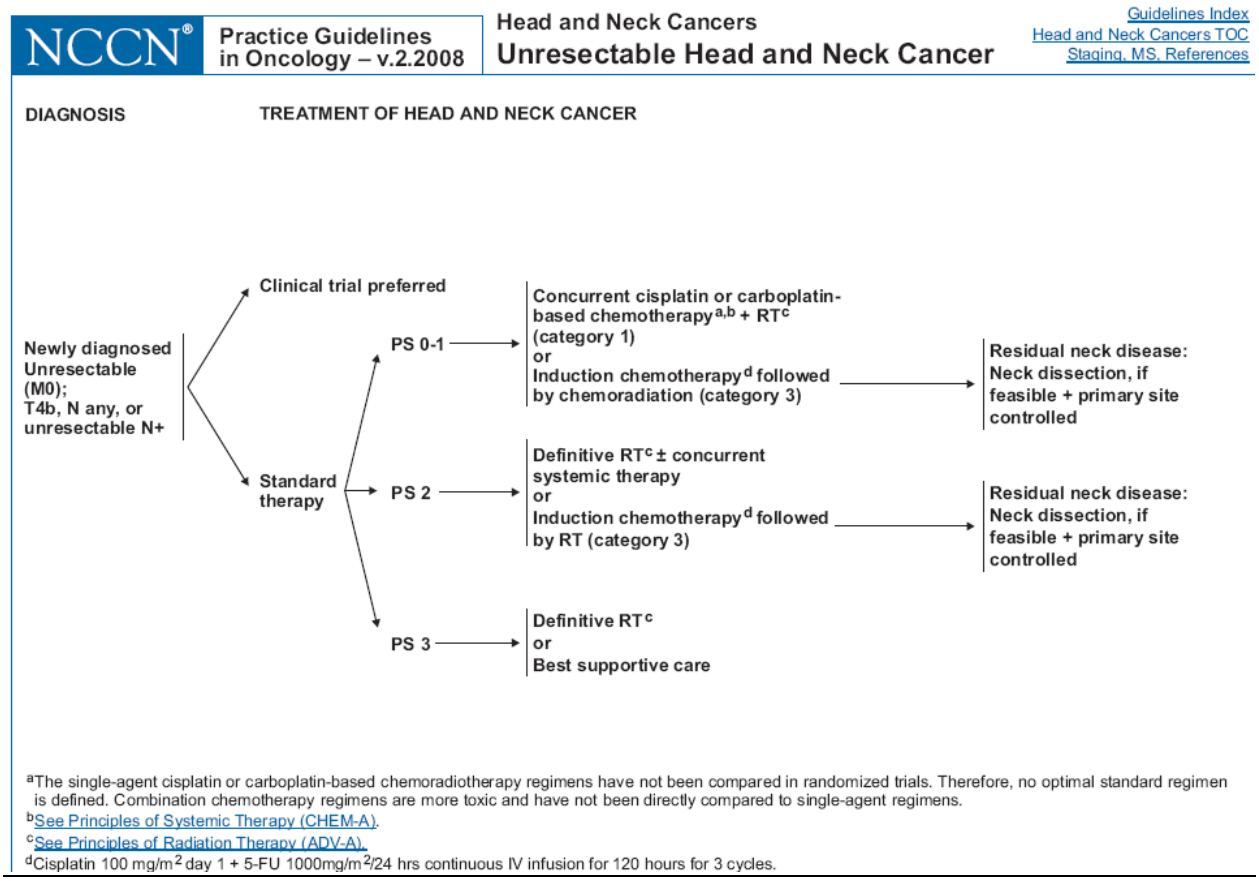
Figure 2



[a]The single-agent cisplatin or carboplatin-based chemoradiotherapy regimens have not been compared in randomized trials. Therefore, no optimal standard regimen is defined. Combination chemotherapy regimens are more toxic and have not been directly compared to single-agent regimens.
[b]See Principles of Systemic Therapy (CHEM-A).
[c]See Principles of Radiation Therapy (ADV-A).
[d]Cisplatin 100 mg/m^2 day 1 + 5-FU 1000mg/m^2/24 hrs continuous IV infusion for 120 hours for 3 cycles.

Figure 3

Systemic Therapy and Radiotherapy according to the NCCN guidelines

PRINCIPLES OF RADIATION THERAPY

Definitive RT
- **Primary and gross adenopathy:**
  ≥ 70 Gy (2.0 Gy/day)
- **Neck**
  Uninvolved nodal stations:
  ≥ 50 Gy (2.0 Gy/day)

Postoperative RT
- **Indicated for pT3 or pT4 primary; N2 or N3 nodal disease, nodal disease in levels IV or V, perineural invasion, vascular embolism.**
- **Preferred interval between resection and postoperative RT is 6 weeks.**
- **Primary: ≥ 60 Gy (2.0 Gy/day)**
- **Neck**
  - **Involved nodal stations:**
    ≥ 60 Gy (2.0 Gy/day)
  - **Uninvolved nodal stations:**
    ≥ 50 Gy (2.0 Gy/day)

Postoperative chemoradiation
- **Indicated for extracapsular nodal spread and/or positive margins [1,2,3]**
- **Consider for other risk features: pT3 or pT4 primary; N2 or N3 nodal disease, nodal disease in levels IV or V, perineural invasion, vascular embolism.**
- **Concurrent single agent cisplatin at 100 mg/m^2 every 3 wks is recommended.**

PRINCIPLES OF SYSTEMIC THERAPY (Page 1 of 2)

The choice of chemotherapy should be individualized based on patient characteristics (performance status, goals of therapy).

Squamous Cell Cancers

Maxillary Sinus, Ethmoid Sinus, Lip, Oral Cavity, Oropharynx, Hypopharynx, Glottic larynx, Supraglottic larynx, Occult Primary

Primary Systemic Therapy + concurrent RT
- Cisplatin alone [1,2] (preferred) [3,4]
- 5-FU/hydroxyurea [5]
- Cisplatin/paclitaxel [5]
- Cisplatin/infusional 5-FU [5]
- Carboplatin/infusional 5-FU [6]
- Cetuximab [7]

Postoperative Chemoradiation
- Cisplatin alone [8,9,10]

Induction chemotherapy
- Docetaxel/cisplatin/5-FU [11,12,13]

Nasopharynx

Chemoradiation followed by adjuvant chemotherapy
- Cisplatin + RT followed by Cisplatin/5-FU [14]

Unresectable/Recurrent Head and Neck Cancers

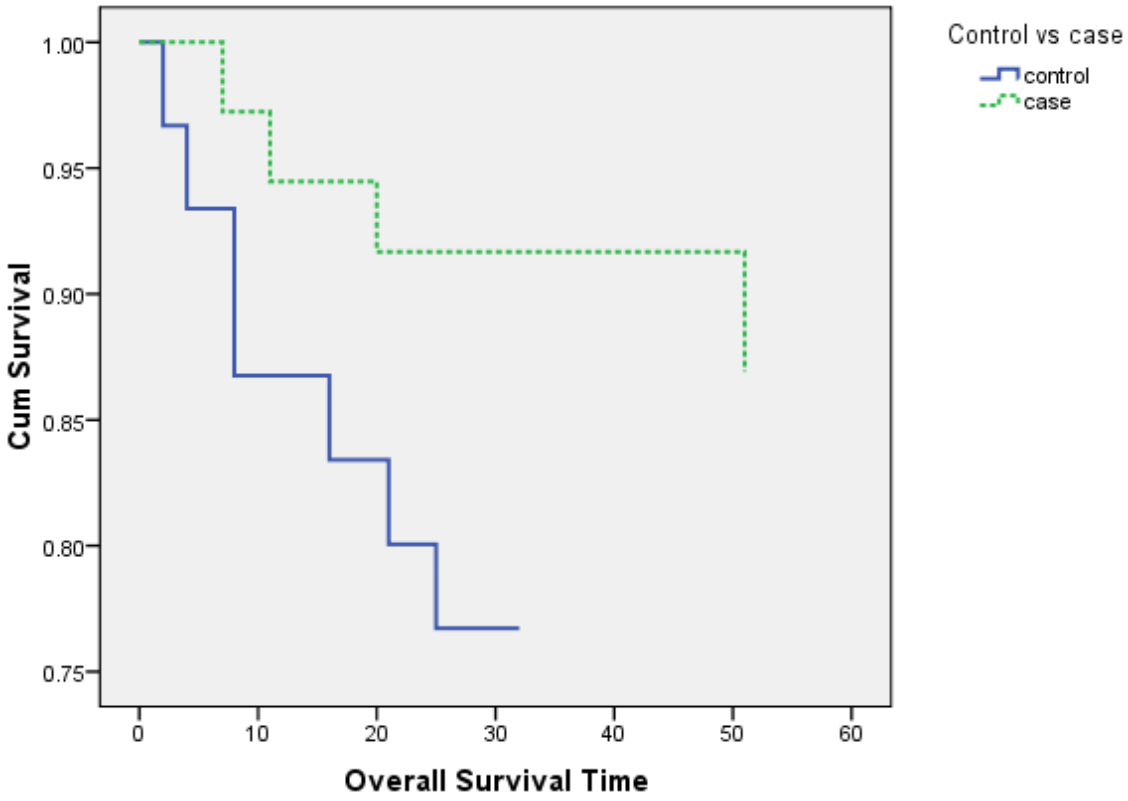
Combination therapy
- Cisplatin or carboplatin + 5-FU [15,16] ± cetuximab [17]
- Cisplatin or carboplatin + docetaxel or paclitaxel [6]
- Cisplatin/cetuximab [18]

Single agent
- Cisplatin
- Carboplatin
- Paclitaxel
- Docetaxel
- 5-FU
- Methotrexate
- Ifosfamide
- Bleomycin
- Gemcitabine [19] (nasopharyngeal)
- Cetuximab [20]

See References on page CHEM-A 2 of 2

Figure 4

Kaplan Meier Overall Survival curves for the two groups (Cases vs. Controls)



p-value = 0.066

Figure 5

Disease free interval curves for the two groups (Cases vs. Controls)

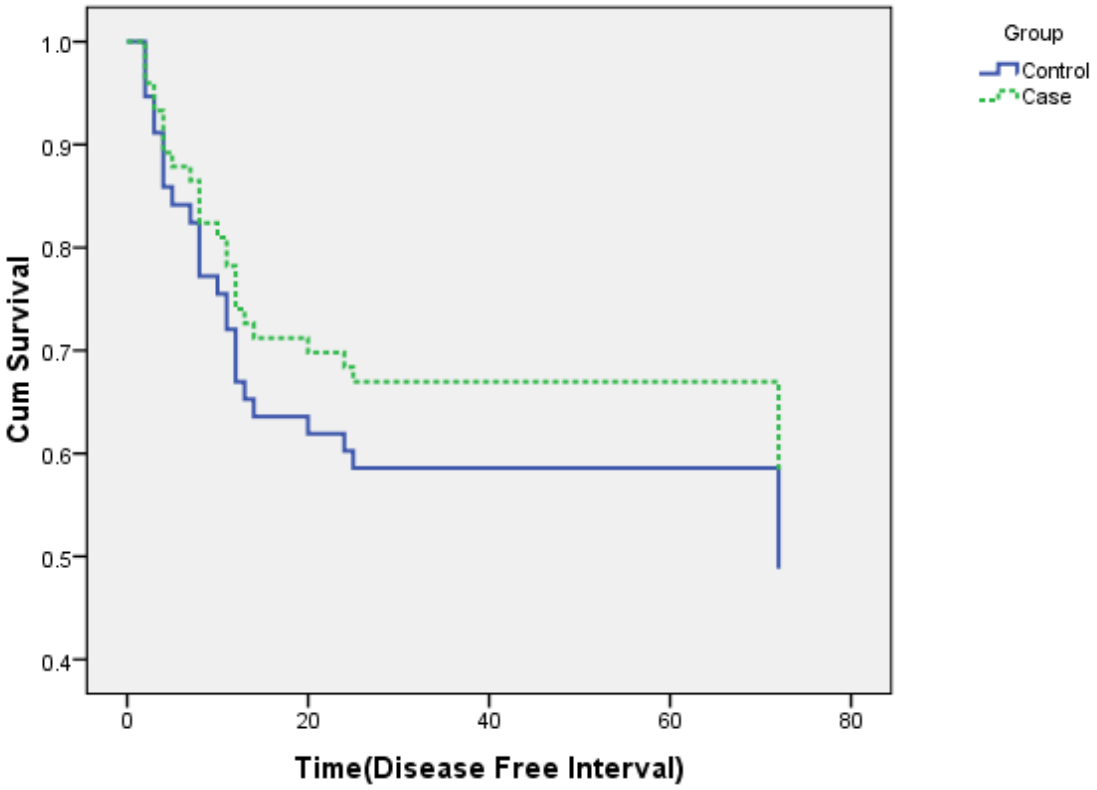
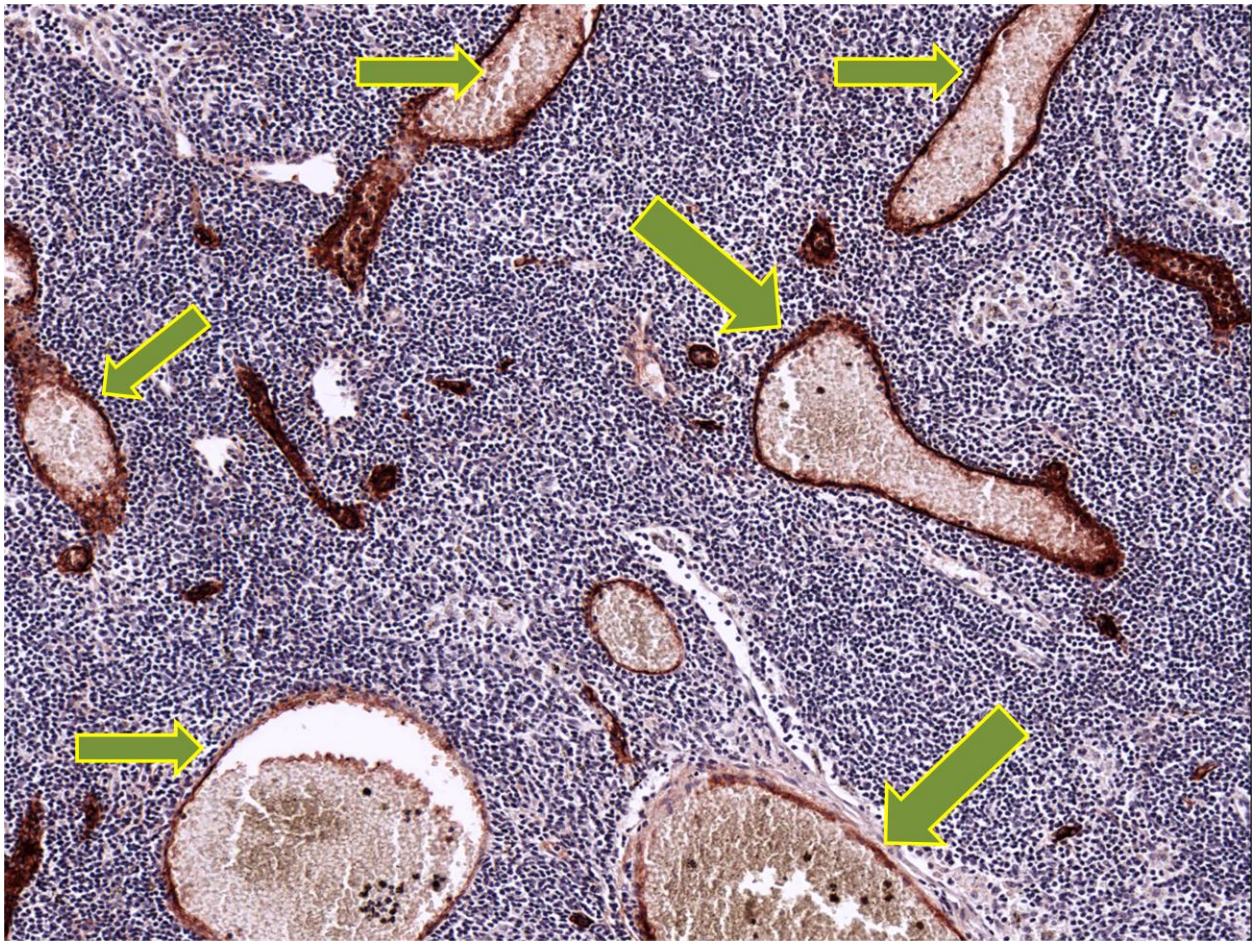


Figure 6

Dilated HEVs with red blood cells in its lumen (high power field)



Green arrows point to the dilated HEVs with red blood cells in its lumen in the lymph node

Figure 7

Metamorphosis of HEVs in a tumor microenvironment.

This process begins with the HEV increasing in absolute numbers, then each of them becoming more dilated and lastly every one of them will become a function vessel carrying blood.

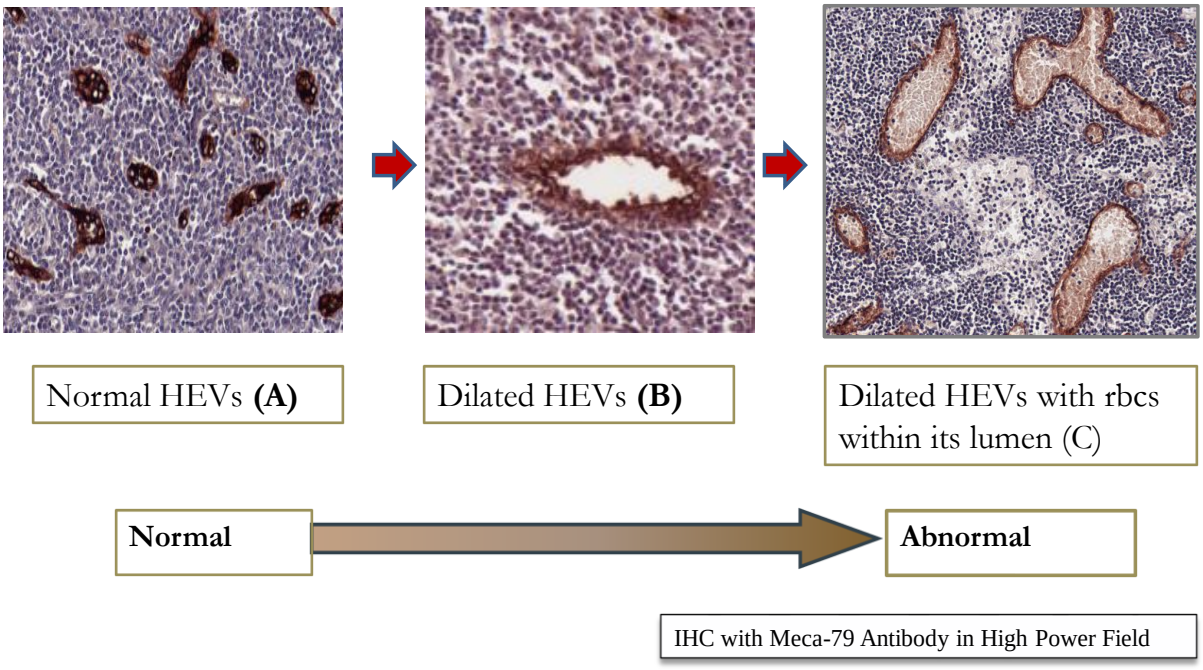
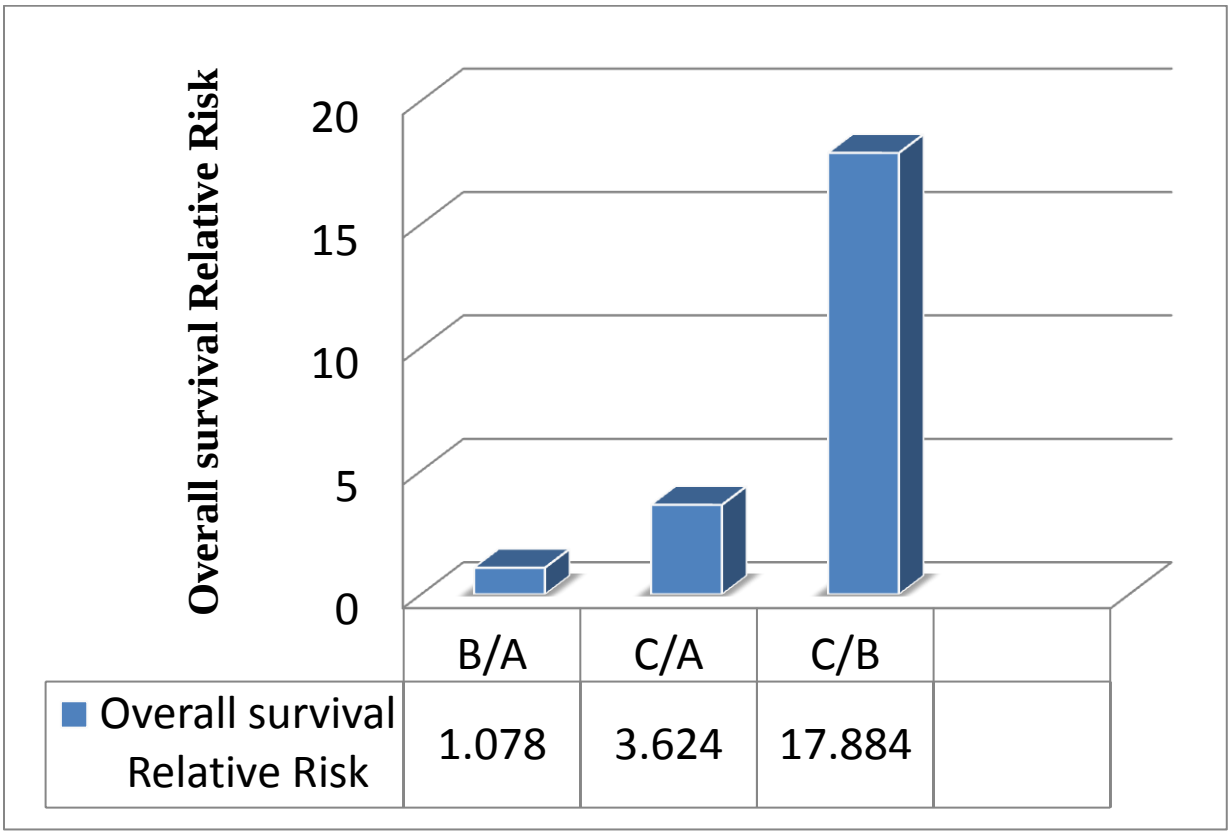


Figure 8

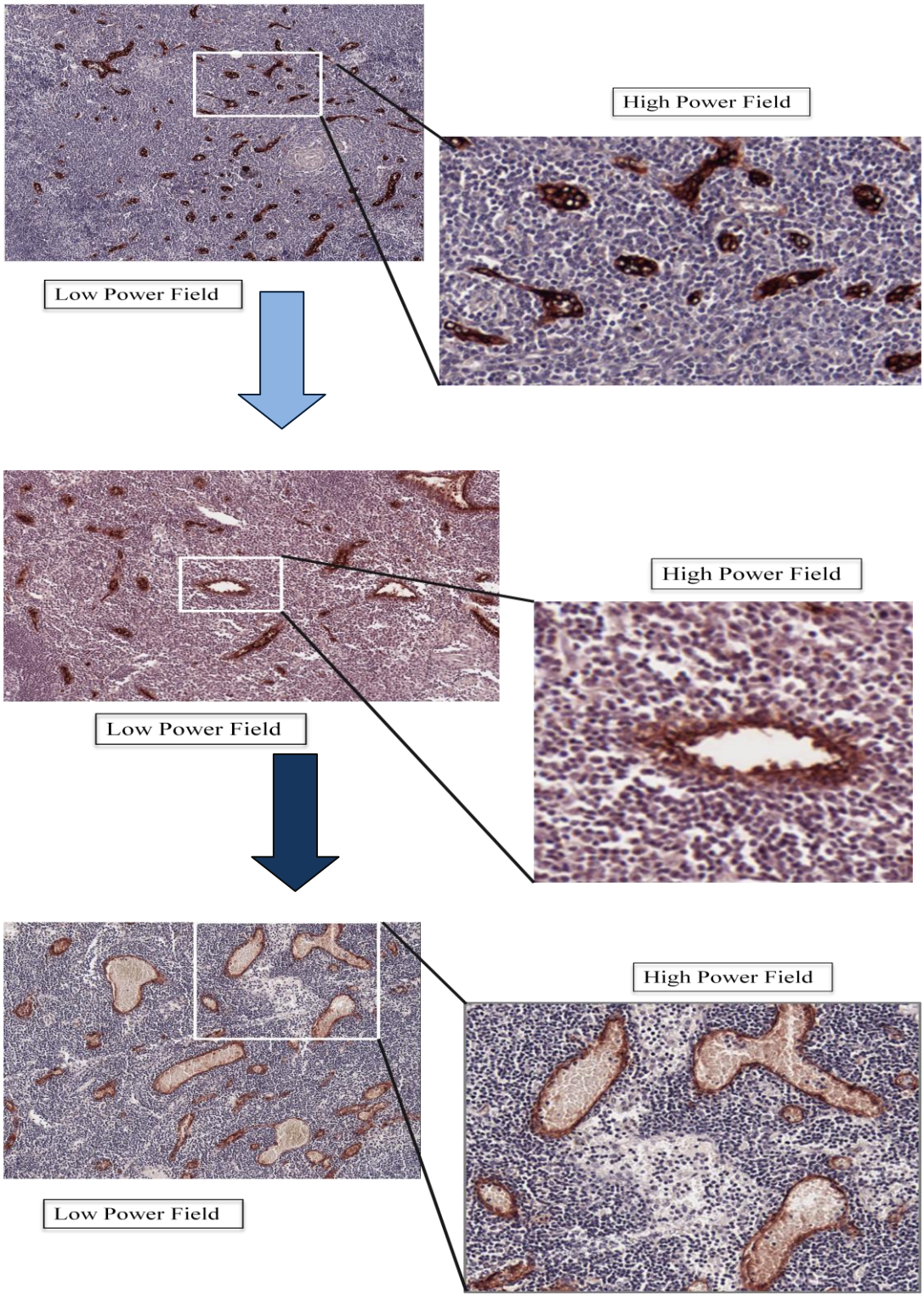
Overall survival relative risk with respect to the different HEVs ratios



- no. of all HEVs : **A**
- no. of dilated HEVs (defined as lumen size more than 80square micron) : **B**
- no. of dilated HEVs with red blood cells (rbcs) inside its lumen : **C**
- Percentage of dilated HEVs with respect to total no. of HEVs i.e. Ratio of dilated HEVs to the total number of HEVs : **B/A**
- Percentage of dilated HEVs with rbcs within its lumen with respect to total no. of dilated all HEVs i.e. Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs : **C/B**
- Percentage of dilated HEVs with rbcs within its lumen with respect to total no. HEVs : **C/A**

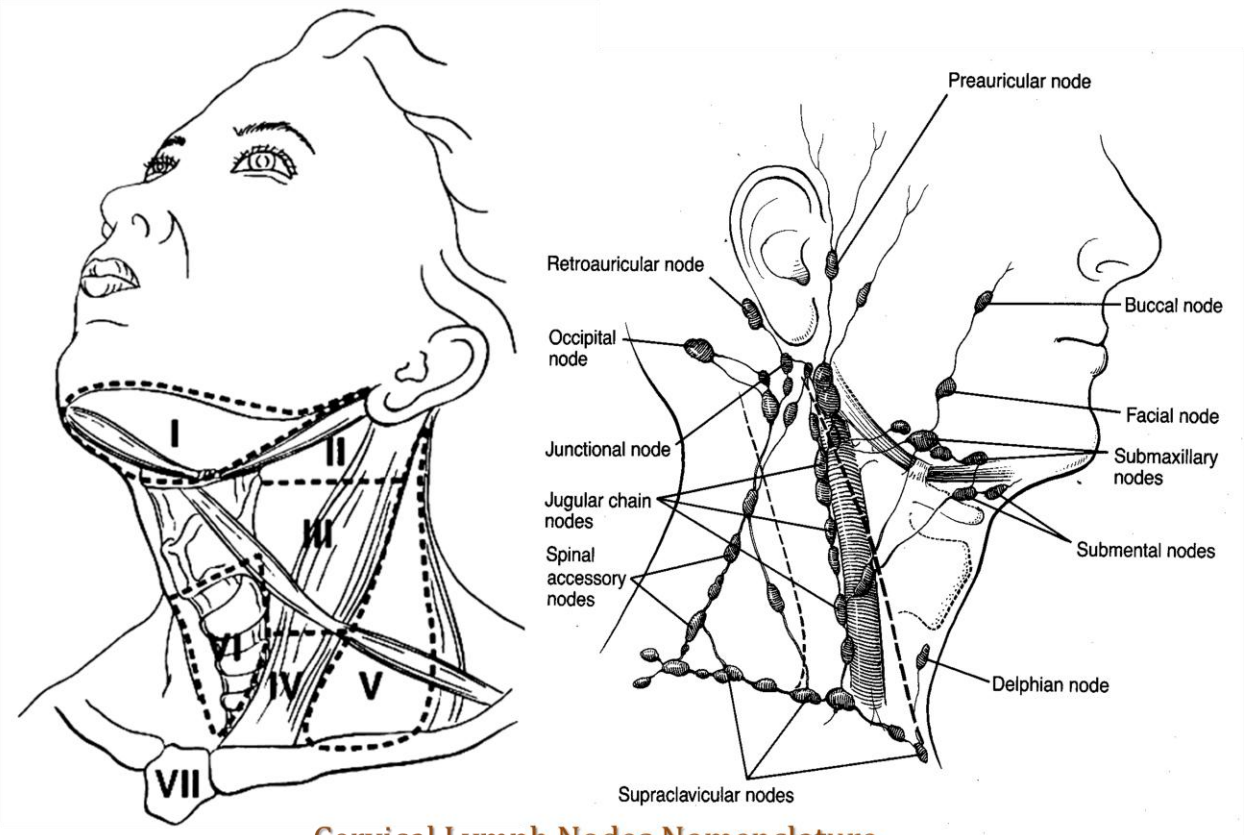
Figure 9

HEV was remodeled from a thick-walled, endothelial vessel with a small lumen to a thin walled, large-lumen vessel



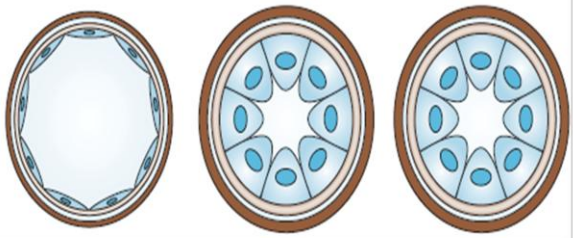
4. List of Illustrations

Illustration 1



Cervical Lymph Nodes Nomenclature

Illustration 2

	Normal venule	Peripheral lymph-node HEV	Peyer's-patch HEV
			
Endothelium	Flat	Tall and plump	Tall and plump
Basal lamina	Thin	Thick	Thick
Perivascular sheath	Scanty	Prominent	Prominent

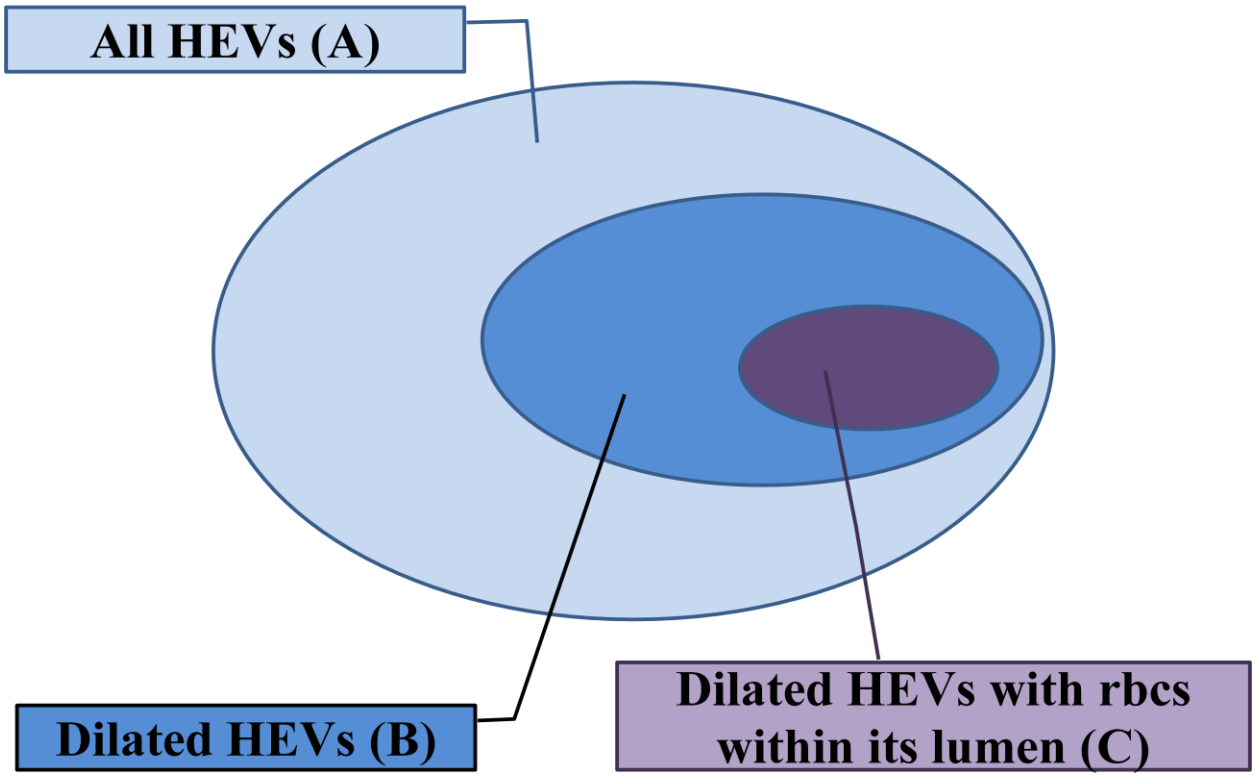
Morphological differences in normal venules, HEVs in LNs, Peyer's patches.

Ref. Miyasaka M and Tanaka T. Lymphocyte trafficking across high endothelial venules: Dogmas and enigmas. Nature Reviews. Immunology 4: 363-370; 2004

Illustration 3

Venn diagram illustrating the relationship between the different HEV parameters (**A, B, C**).

(I.e. how B is a subset of A and C is a subset of B.)



5. Introduction and Background

Cancer remains one of the leading causes of morbidity and mortality worldwide. Oral and pharyngeal cancers is ranked as the eighth most common cancer diagnosed in men in the United States (1) . Despite advances in surgery and radiation therapy, the 5-year survival rate for oral cancer has not improved significantly over the past several decades and remains at 50–55% (2, 3) . This is primarily because patients continue to die from metastatic disease at regional and distant sites as well as from local recurrence.

Cancer research has focused a great deal on the pathogenesis of metastasis as the presence of metastases often translate to a grave prognosis with relatively little effective therapeutic measures currently available, in contrast to early non-metastatic lesions.

Dissemination of primary malignant cancer cells is traditionally described as being via several routes. Firstly, by direct local invasion into the surrounding tissue or transcoelomic spread by seeding of the cancer cells into body cavities e.g. peritoneal, pleural surfaces. Secondary, via systemic metastasis via tumor-associated blood vessels to distant organs and or, lymphatic metastasis via tumor associated lymphatic vessels to draining sentinel lymph nodes (SLN), then to distal lymph nodes, and from there to distal organs. Lastly, via transplantation, mechanical deposition or spillage of cancer cells by surgery or use of instruments during diagnostic procedure e.g. biopsy.

Sentinel lymph node metastasis is the initial step in the spreading of cancer in many malignancies. The first lymph node was called the ‘‘sentinel node’’ by Cabanas in 1977, studying penile cancer, who defined the concept of the sentinel node being the doorway to the

regional node basin (4). The modern concept of SLN represents the group of LN in the first draining station, this usually represents about 2 to 6 nodes in each echelon. This assumption has now been firmly established in breast cancer, melanoma, and other cutaneous sites such as vulvar and Merkel cell cancers but is less well defined in others such as thyroid, head and neck, gastric, colorectal, cervical, and endometrial cancers.

The sentinel or the regional lymph nodes undergoes morphological and functional changes induced by the primary tumor. These are reflected and may be brought into effect by vasculature and lymph channel reorganizations even before the arrival of cancer cells. The key blood vessels in such lymph nodes that are remodeled are identified as high endothelial venules (HEV) (5) . Tumor-reactive lymphadenopathy in SLNs has been observed for decades, but alterations of the lymphatic channels and vasculature in these nodes before the arrival of metastatic tumor cells remain largely unexplored and not well characterised.

6. Squamous Cell Carcinoma of the Tongue

6a. Epidemiology

Oral and pharyngeal cancers is ranked as the eighth most common cancer diagnosed in men in the United States (1) . It is estimated that about 35,310 new cases of oral cavity and oropharyngeal cancer will be diagnosed in the United States in 2008; 25,310 in men and 10,000 in women. An estimated 7,590 people (5,210 men and 2,380 women) will die of these cancers in 2008 (6) . As the incidence of oral cancer continues to increase, the disease becomes an increasingly important public health issue. The World Health Organization (WHO) predicts a continuing worldwide increase in the number of cases of oral cancer for the next several decades (7) . It was indicated that oral cancer in Europe constituted between 25% to 35% of all cancers then (7) .

Of all the carcinomas of the head and neck, tongue is the most prevalent site. The results of numerous studies suggest that head and neck cancer, particularly oral tongue cancer, is increasing in young adults internationally (8-14) . This may be attributed to the higher incidence of young people picking up smoking and the increasing incidence of HPV infection in young adults.

In the United States, an increase in the tongue cancer mortality rate in adults younger than 30 years has been described (12). Oral tongue cancers are also associated with an increased proportion of female patients, non-smokers and aged <40 years (15) . It has also been reported worldwide to be a raising cause of mortality in males (7) .

6b. Clinical and Pathological features

Oral tongue SCC was associated with poorer survival compared with other oral cavity and head and neck sites (11, 12, 16, 17) .

The oral tongue is the oral cavity subsite associated most commonly with squamous cell carcinoma (SCC). Previous studies have demonstrated that cancers of the oral tongue are distinct biologically and epidemiologically from other tumors of the oral cavity (15) .

There are many risk factors associated with oral cancer including tongue cancer. There are commonly known as the 6 "S", namely, **s**moking, **s**pirits(alcohol), **s**ex, **s**yphilis, **s**unlight, exposure (lip carcinoma), immunosuppressed **s**tates (AIDS, post-transplant patients). About 90% of people with oral cavity and oropharyngeal cancers use tobacco, and the risk of developing these cancers is related to the duration and the amount of tobacco they smoked or chewed. Tobacco smoke from cigarettes, cigars, or pipes is associated with the increased incidence of cancer in general and they arise from anywhere in the oral cavity and oropharynx, lungs, esophagus, kidneys, bladder, and several other organs. Oral tobacco products usage is also associated with cancers of the oral mucosa and inner surface of the lips.

Alcohol consumption, in addition to smoking strongly increases a person's risk of developing oral cavity and oropharyngeal cancers. About 70% with oral cancer are heavy alcohol drinkers. People who are particularly heavy alcohol drinkers but don't smoke still retain a high risk of these cancers, but it is the combination of the alcohol consumption and tobacco usage that is the most dangerous.

Oral and oropharyngeal cancers are about twice as common in men as in women. This may attributed to the common fact that men are more likely to use tobacco and alcohol. This difference is decreasing as more women are now using tobacco and drinking nowadays.

Nutritional factors may play a role as well. Several studies have found that a diet low in fruits and vegetables is linked with an increased risk of cancers of the oral cavity and oropharynx. This may be confounded by the poorer dentition and prevalence of poorer general dental health and hygiene in this population of lower social economic status.

Human papilloma viruses (HPV) are a group of more than 100 related viruses. Most HPV types and benign and cause viral warts on various parts of the body, but a few HPV types seem to be involved in some cancers. Cancer of the cervix is the most notorious cancer to be associated with many strains of HPV. The same HPV types (especially HPV strain 16) are found in some oral and oropharyngeal cancers (18) . The current view is that HPV may be a factor in the development of up to a third of oral and oropharyngeal cancers. People with oral cancer linked with HPV infection are less likely to be smokers and drinkers, and in general seem to have a better outlook than those without HPV. HPV-positive tumors have a better clinical outcome and prognosis.

Immunosuppression is known to be associated with cancer development e.g. people with Acquired Immunodeficiency Disease (AIDS) are known to be at risk of developing Kaposi sarcoma. Drugs that suppress the immune system to prevent rejection of transplanted organs or

to treat certain immune system diseases may be at increased risk for cancers of the oral cavity and oropharynx.

Lichen Planus is a skin condition that occurs mainly in middle-aged people. Most often it affects the skin, presenting as an itchy rash but it sometimes affects the oral and oropharyngeal muscoa, appearing as small white lines or spots. A severe case may slightly increase the risk of oral cancer.

There are many other unproven and controversial risk factors reported in the literature. The common mouthwash has high alcohol content, it has been suggested to be linked with a higher risk of oral and oropharyngeal cancers. Studies researching this possible association are plagued by the confounding fact that most smokers and frequent are more likely to use mouthwash than people who neither smoke nor drink. It has been suggested that long-term irritation of the oral lining caused by poorly fitting dentures is a risk factor for oral cancer. This has yet to be proven unequivocally.

6c. Current Opinions on management and therapy of Squamous Cell Carcinoma of the Tongue

The tongue is the most common intraoral site of cancer in most countries. The oral tongue is the site in oral cavity associated most commonly with squamous cell carcinoma (SCC).

Clinical Presentation

Cancer of the tongue may grow to significant size before they cause symptoms. Approximately three quarters of the cancer occurs in the mobile tongue and most are well differentiated tumors. Tongue cancer may spread easily because the tissue planes separating the intrinsic tongue musculature are lax. They often become symptomatic when its size interferes with movement causing speech or swallowing problems or when they cause pain. Squamous cell carcinoma of the tongue may arise in apparently normal epithelium, in areas of leukoplakia or in an area of chronic inflammation e.g. chronic glossitis. These lesions are often larger than 2 cm at presentation, with the lateral border being the most common subsite. At an advanced stage, the patient may develop speech and swallowing dysfunction. Pain can sometimes occur when the tumor involves the lingual nerve and this pain may also be referred to the ear as a result. Carcinomas of the tongue base are clinically silent until they deeply infiltrate the tongue musculature. They are often less differentiated. As a result of a relative asymptomatic early stage combined with the difficulties with direct visualization, they may extend into the oral tongue or have clinical lymph metastases before the diagnosis is established.

The evaluation of a patient with tongue cancer begins with a detailed history and a complete head and neck clinical examination. History includes specifically asking about tobacco and alcohol use and quantifying its usage, pain, weight loss, articulation difficulties, referred otalgia, hemoptysis, hoarseness, and dysphagia and odynophagia. During a complete head and neck examination, attention is directed at the site and size of the lesion and any infiltrating characteristics; a thorough bimanual examination of the tumor, the surrounding floor of mouth, and the submandibular triangles is performed. Lymphadenopathy is carefully examined with the palpation of the bilateral neck and this is completed with a full dental evaluation, with attention to dental hygiene, dentition status, and integrity of the mandible. A sample of the lesion e.g. punch biopsy may be obtained in the clinical setting or as part of the endoscopic evaluation of the tumor to obtain pre-operative confirmatory histology. If indicated, Examination under Anesthesia (EUA) may be helpful especially for posterior or base of tongue cancers (19) .

A comprehensive whole body examination is also performed with attention to assessing the fitness for surgery or adjuvant therapy and to exclude metastatic disease. The clinical evaluation is completed with appropriate imaging modalities e.g. Computed Tomography (CT) or Magnetic Resonance Imaging (MRI).

Imaging Investigations

Radiologic evaluation with a CT scan and MRI has revolutionized the assessment of patients with head and neck tumors. An MRI has higher soft tissue resolution and the assessment of the mobile tongue may be facilitated. Involvement of the extrinsic tongue musculature and direct extension in the submandibular glands and the base of tongue can be revealed with MRI. The

response to therapy also may be evaluated more thoroughly. As part of the staging and management processes, confirmation of nodal disease, vascular distortion or involvement, bony destruction, or potential space involvement aids in the diagnosis.

Both CT scan and MRI are generally reliable for detecting the extent of soft tissue and bony involvement in persons with oral cavity carcinoma (20) . However, MRI has several well established advantages in staging tumors of the oral cavity. The soft tissue contrast between tumor and normal musculature is higher on T2-weighed images. With MRI, there is minimum or no beam artifact from amalgam or other dental material. Imaging with MR can be performed and reconstructed in sagittal, coronal, and axial planes giving the surgeon a 3-Dimension impression for a more accurate tumor assessment and this aids in surgical planning. The contrast between post-irradiation fibrosis and recurrent tumor is also better appreciated on T2-weighed images.

Recently, with the advent of positron emission tomography (PET), combining PET and CT is a new diagnostic and staging modality in the evaluation of the patient with head and neck cancer. PET scans are used most often to reveal cancer and to examine the effects of cancer therapy by characterizing biochemical changes in the cancer. These scans can be performed on the whole body or can be localized to the head and neck.

A PET scan demonstrates the biological changes in the tissues or organs before anatomical changes take place by utilizing the radiotracer 18-fluorodeoxyglucose (FDG), while the CT scan provides information about the body's anatomy, such as size, shape, and location. Squamous cell carcinomas and many other malignant tumors demonstrate increased glucose metabolism as

compared to normal tissues. [18F]-fluoro-2-deoxy-D-glucose is a glucose analogue that may be delivered intravenously and preferentially transported into squamous cell carcinomas by glucose transporters By combining these 2 scanning technologies, a PET-CT scan enables physicians to more accurately diagnose and identify cancer and its extent. These can be used as a tool in the initial evaluation of the patient who presents for initial staging, as well as for evaluating response to treatment and detection of recurrences (21) . The major disadvantage of PET scan currently like all new technologies is the lack of availability and the high operating and set-up cost of the procedure.

Laboratory Investigations

There are no established biochemical tumor markers for tongue cancer. The incidence of distant metastases at initial presentation is low and therefore the only laboratory workup needed is directed at the evaluation of the patients' underlying medical conditions and assessing the patient's fitness for surgery. A full blood cell count and a biochemical panel of urea, creatinine and electrolytes is a useful general screening tool A Chest X-ray (CXR) and 12-lead Electrocardiogram (ECG) are standard pre-operative investigations for any person undergoing major surgery, general anesthesia or aged above 40 years old. Usually for patients undergoing major surgery or in patients with a history or suspected bleeding diathesis, investigations may also include tests of prothrombin time (PT), partial thromboplastin time (PTT), and international normalized ratio (INR). These tests will help the primary physician to establish if further testing is warranted.

Treatment

The treatment approach is best managed by a multi-disciplinary team. The role of clinical evaluation with history taking, physical examination and imaging is to help guide treatment options by staging the cancer using the American Joint Committee on Cancer (AJCC) TNM Staging System (22) . (Table 1)

Early lesions (T1 and T2) of the tongue may be managed by surgery or by radiation therapy (RT) alone. Both modalities produce 70% to 85% cure rates in early lesions. Moderate excisions of tongue, even hemiglossectomy, can often result in surprisingly little speech disability provided the wound closure is fashioned such that the tongue is not bound down. If, however, the resection is more extensive, problems may include aspiration of liquids and solids and difficulty in swallowing in addition to speech difficulties. Occasionally, patients with tumor of the tongue require almost total glossectomy. Larger lesions generally require combined surgical and radiation treatment. The control rates for larger lesions are about 30% to 40%. More advanced lesions may require segmental bone resection, hemi-mandibulectomy, or maxillectomy, depending on the extent of the lesion and its location.

Generally, surgery if possible is the preferred main stay of treatment and offers the best option if there are no contraindications. Treatment with surgery alone for early or superficial lesions or in combination with adjuvant radiotherapy for more advanced lesions is the standard of care. For tongue cancers and all SCC of the head and neck, the neck needs to be considered as part of the treatment. The cervical nodes can be treated with either surgery, radiation therapy or both. Major advances have been made in surgical approaches, reconstructive options, and the rehabilitation of patients who have oral cavity and tongue SCC. These advances have significantly improved

disease-specific outcome and quality of life. The therapeutic decision must take into consideration the patient's age, lifestyle, and willingness to participate in the therapeutic regimen. The treatments have substantially different morbidities and may result in significant differences in quality of life.

The treatment protocol according to the Stage is detailed below and summarized in Figure 3. The treatment guidelines according to the internationally well- accepted National Comprehensive Cancer Network (NCCN) recommendations are classified into several groups:

1. T1 or T2, N0
2. T3, N0
3. T1 to T3, N1 to N3; T4a, any N
4. Unresectable.

1. <u>Treatment guidelines for T1 or T2, N0 tumors</u>

   The preferred option is excision of the primary tumor with or without a unilateral or bilateral selective neck dissection. The decision of whether a neck dissection is done depends on clinical suspicion and judgment on the risk of occult cervical LN metastasis. Generally, if the risk deemed to higher than 20%, most institutions would elect a form of neck dissection. Bilateral neck dissections are performed based on clinical judgment and if the lesion is central or near the midline. Recently intra-arterial chemotherapy combined with radiotherapy has been shown to have good organ preservation and reasonable therapeutic results.

After surgery, the treatment is stratified according to the presence or absence of adverse features. (Table 2) If there are no adverse features noted, no adjuvant therapy is needed. If there is one positive LN without adverse features, RT is an option to be considered. If adverse features of ECS and/or positive margins, Chemo/RT is recommended. If there are any other risk factors present, RT alone or Chemo/RT are the options.

The other option of primary treatment is External-beam RT with or without brachytherapy. If there is evidence of residual disease, salvage surgery is recommended.

2. Treatment guidelines for T3, N0 tumors

The guideline is excision of the primary tumor and reconstruction as indicated and a unilateral or bilateral selective neck dissection. If there are no adverse features noted, RT is optional. If adverse features of ECS and/or positive margins, Chemo/RT is recommended. If there are any other risk factors present, RT alone or Chemo/RT are the options.

3. Treatment guidelines for T1 to T3, N1 to N3 tumors; T4a, any N tumors

Surgery is the mainstay of treatment. In this group of patients, choice of surgery is dependent on the nodal status.

For N0, N1, N2a, N2b, N3 tumors, the recommendation is excision of the primary tumor and an ipsilateral comprehensive with or without a contralateral selective neck dissection and reconstruction as indicated.

For N2c (i.e. bilateral or contralateral LNs metastasis) tumors, the recommendation is excision of the primary tumor and a bilateral comprehensive and reconstruction as indicated.

Adjuvant therapy recommendation is the same, i.e. If there are no adverse features noted, RT is optional. If adverse features of ECS and/or positive margins, Chemo/RT is recommended. If there are any other risk factors present, RT alone or Chemo/RT are the options.

4. Treatment guidelines for unresectable tumors

Any newly diagnosed unresectable; T4b; N+ unresectable tumors, the standard treatment options are:

a. Concurrent Cisplatin or Carboplatin-based chemotherapy + RT (Category 1 evidence) (Figure 2)
b. Clinical Trial if available
c. Definitive RT plus concurrent systemic chemotherapy. (Category 3 evidence)
d. Induction chemotherapy followed with chemoRT. (Category 3 evidence)
e. Induction chemotherapy followed with RT. (Category 3 evidence)
f. Definitive RT. (Category 2a evidence)
g. Best Supportive Care

Systemic Therapy and Radiotherapy

Systemic Therapy and Radiotherapy are reserved for advanced HNSCC. The treatment of locoregionally advanced squamous cell carcinoma of the head and neck has evolved gradually from surgery as the mainstay of treatment to radiotherapy as the principal treatment. Additional benefit has been obtained with hyperfractionated radiotherapy and with radiotherapy combined with chemotherapy (chemoradiotherapy). The value of chemoradiotherapy is, however, counterbalanced by increased and often prohibitive toxicity, particularly among patients with coexisting medical conditions and decreased performance status.

A multinational, randomized study in 2006 by Bonner et al. studied Radiotherapy alone versus Radiotherapy plus cetuximab (Erbitux, ImClone Systems), an IgG1 monoclonal antibody against epidermal growth factor receptor (EGFR), in the treatment of loco-regionally advanced squamous -cell carcinoma of the head and neck. The addition of cetuximab to high-dose radiotherapy significantly increased both the duration of control of locoregional disease and survival among patients with locoregional advanced head and neck cancer. These benefits were achieved without the prohibitive in-field toxic effects often associated with high- dose radiotherapy to the head and neck (23) .

The principles and details of Systemic Therapy and Radiotherapy according to the NCCN are summarized in Figure 3.

Follow-up

Patients with treated tongue or oral cavity cancers are followed up on periodic physical examination, 1 to 3 monthly in the first year; 2 to 4 monthly in the second year; 4 to 6 monthly in year 3 to year 5 and after 5 years, 6 months to yearly. During the follow-ups, chest imaging as clinically indicated. Speech, hearing and swallowing evaluation and rehabilitation as indicated. Smoking cessation and dental followed is also recommended.

6c i. Role of Neck Dissection in the surgical management of tongue cancer

The head and neck encompasses perhaps some of the most anatomically complicated regions of the body. Good knowledge of the lymphatic system is essential to understand the pattern of spread of cancer in the neck. About a third of all the lymph nodes in the body are in the head and neck region. Most squamous cell carcinomas of the head and neck are at least potentially curable if the neck is appropriately treated as well, as the risk of occult metastasis in tongue cancer can be up to 20% to 40%. Metastasis to cervical lymph nodes occurs more frequently from the tongue than from any other primary tumor site in the oral cavity.

The LNs in the cervical region are described by levels that correspond to their anatomic location. (I-VII). The LNs in the submental and submandibular triangles make up level I, and the upper, middle, and lower jugular nodal groups are considered levels II, III, and IV, respectively. The posterior triangle of the neck is designated as level V, while the pre-laryngeal, pre-tracheal, and para-tracheal nodes comprise level VI. Level VII includes the lymph node groups found in the upper mediastinum. (Illustration 1)

The tongue has a dense lymphatic network, with three main deep muscular lymphatic drainage pathways. The anterior pathway drains the tip of the oral tongue, primarily to level I or level II. The lateral pathway drains the lateral 1/3 of the dorsum of the tongue to levels I, II, III. The central pathway drains the central 2/3 of the tongue. These vessels drain to the level I nodes or course through a sublingual node and terminate in the level III nodes. Most of the lymph from the oral tongue drains to levels I and II. The levels most frequently involved with single pathologic lymph node metastasis in SCCOT are levels I, II, and III (24) .

The prognostic value and significance of metastatic disease in the cervical lymph nodes of head and neck cancer has long been appreciated and well established in many studies. Since the 19th century, surgeons have attempted to remove involved cervical lymph nodes at the time of resection of the primary cancer, a systematic approach to en bloc removal of cervical lymph nodes. Radical en bloc Neck Dissection has been described by a Polish surgeon, Jawdyn´ski in 1888 and subsequently popularized by American Surgeon Dr. George W. Crile in the 1906. Dr. George W. Crile of Cleveland, Ohio has since been regarded in North America as the "Forefather" of head and neck cancer surgery, akin to what Dr. William Stewart Halsted is to breast surgery. These pioneers' work provided the basis of our current techniques. Washington University in 1933 described their experience in 131 cases and advocated the removal of the spinal accessory nerve during neck dissection as it facilitated a more complete removal of the cervical lymph nodes and shortens operative time (25) . Over the next few decades, developments included preservation of the spinal accessory nerve in selected cases, elective neck dissection performed in association with resection of various primary tumors, bilateral neck dissection and limited neck dissection were reported and practiced with varying results. In 1963,

the first description of an effective technique of modified radical neck dissection was published in Spanish by Sua´rez, in 1963. His technique preserves three important structures, the internal jugular vein, sternocleidomastoid muscle and spinal accessory nerve, this technique was further refined and modified by various authors who published their results in the English language literature during the period from 1964 through 1990 and beyond. Modified or ‘‘functional’’ neck dissection gains popularity because the technique avoids much of the morbidity of radical neck dissection while achieving equivalent degrees of control of regional disease in properly selected cases. By the late 20th century, the concept of selective neck dissection, consisting of resection of only the nodal groups at greatest risk for metastasis from a given primary site, was studied and developed.

Due to various techniques and the lack of standardization, in 1988, the Committee for Head and Neck Surgery and Oncology of the American Academy of Otolaryngology-Head and Neck surgery (AAO-HNS) convened a committee to standardize the nomenclature. This is updated recently and confirmed by the Committee for Neck Dissection Classification, American Head and Neck Society; representation from the Committee for Head and Neck Surgery and Oncology, American Academy of Otolaryngology–Head and Neck Surgery (T.A.D.) (26) . They defined the following nomenclature for neck dissections (ND).

1. Radical ND, considered to be the standard procedure for cervical lymphadenopathy
2. Modified radical ND, this encompasses any alteration of the radical procedure that involves preservation of one or more non-lymphatic structures of the neck.

3. Selective ND, this terms includes supraomohyoid (SOHND), posterolateral, lateral and anterior NDs, each representing a specific procedure that preserves one or more lymph node groups routinely removed in radical neck dissection.
4. Extended ND, or any alteration involving removal of additional lymph node groups or non-lymphatic structures relative to the radical procedure

Newer or other nomenclature in the literature includes comprehensive neck dissection, it is one that removes all the LN groups as one would in a radical neck dissection, whether the internal jugular vein, sternocleidomastoid muscle and spinal accessory nerve are preserved does not affect whether the dissection is comprehensive or not. Similar to MRND, Functional ND, describes removing the entire lymphatic system of the neck by dissecting the fasciae surrounding the non- lymphatic structures, thus preserving the sternocleidomastoid, internal jugular vein, and accessory spinal nerve.

Prospective studies have demonstrated similar rates of neck recurrence and survival after elective selective neck dissection compared to elective modified radical neck dissection. Other modifications and factors applied to treatment of cervical lymph node disease include the use of adjuvant and neo-adjuvant radiation and chemotherapy, the identification of various adverse prognostic factors such as extracapsular spread and extranodal soft tissue deposits, application of sentinel lymph node biopsy to staging of the neck, the use of immunohistochemical and molecular techniques for identification of lymph node metastases not detectable by light microscopy, and the recent possibility of endoscopic neck dissection (27) .

6c ii. Role of Sentinel Lymph Node Biopsy in Oral Tongue Cancers

The sentinel lymph node biopsy (SLNB) derives from a concept pioneered more than 40 years ago (28) . In 1977, Cabanas studying penile carcinoma, using the experience from a hundred cases, he defined the concept of the sentinel node being the doorway to the regional node basin (4) . Since then, with this assumption and belief that the SLN's histological status is representative of its LN basin, the procedure has been gaining momentum and greater interest from the scientific community. Breast cancer and melanoma are the two pathological states in which the SLNB has been most established and validated and thus widely used.

Sentinel lymph node biopsy (SLNB) is a minimally invasive technique that allows the surgeon to excise the primary draining lymph nodes, in order for the pathologist to examine and determine the presence of subclinical metastases with the aid of frozen sections histopathology intra-operatively. The procedure is performed with the assistance of radio-labelled dye injection and/ or lymphoscintigraphy. This technique offers a less invasive technique to stage the lymphatic basins of a patient with a primary malignancy and allows detailed examination of the first echelon lymph node basin for metastases. Sentinel lymph node biopsy techniques have also evolved and improved with time and technological advances, recently some papers has shown the usefulness and potential of Single Photon Emission Computed Tomography and Computed Tomography (SPECT-CT) to increase the accuracy of pre-operative anatomical mapping (29, 30) .

Sentinel lymph node biopsy is currently used in many studies on a trial and experimental status for validation in many cancers e.g. colorectal, gastric , cervical carcinomas. In tongue cancers and head and neck squamous cell carcinoma (HNSCC), the pathologic status of the regional lymph nodes remains the most important disease prognosticator. Head and neck squamous cell carcinoma is often considered a loco-regional disease and the incidence of distant metastasis is relatively low, whereas the incidence of occult loco-regional disease i.e. occult metastasis in cervical LNs even in early stage Tongue SCC can be up to 20% to 30% (31) . This is in contrast to cancers such as melanoma or breast cancer in which lymph node staging is a more accepted indicator of distant metastasis. In the past before the age of CT and MRI, clinical examination has been the mainstay for determining the presence of LN metastasis. Naturally because of the inconsistency and unreliability of palpation alone and with the invention of CT and MRI, physicians have utilized these imaging modalities to detect the presence of nodal metastasis. However, even now, the current imaging tests have been shown to be not reliable in the detection of early nodal disease.

Sentinel lymph node biopsy is currently used as a staging procedure in HNSCC at some European centers and is under active investigation in the United States in an ongoing American College of Surgeons Oncology Group (ACOSOG) trial (32, 33) .

In a multicentre prospective trial, Ross et al. reported their preliminary results. They performed 227 SNB procedures, 93% of the time, a sentinel node was identified. Of 59 positive nodes, 57 were identified with the intra-operative gamma probe and 44 with blue dye. They upstaged patient's diseases in 34%. The sensitivity of the technique was 93%. The identification of SLNB

for floor of mouth (FOM) tumours was 86%, compared with 97% for other tumours. The sensitivity for FOM tumours was 80%, compared with for other tumor groups. They concluded that SLNB can be successfully applied to early tumours of the oral cavity and oropharynx in a standardized fashion by centres worldwide and for the majority of these tumours this technique can be used alone as a staging tool (33) .

Rigual et al. reported in a small prospective study looking at oral carcinoma and the role of SLNB. They identified SLNs in all their patients (100%) and accurately predicted the pathologic nodal status in 90 % of their patients (90%). Occult nodal metastases were present in 60% of the cohort. They reported sensitivity and specificity of SLNB in their patient population of 83% and 100%, respectively, with that they conclude that SLNB is technically feasible and accurate but they also mentioned that sentinel node biopsy can be more technically demanding in the head and neck region especially tumours at the FOM, than in other anatomical sites due to the proximity of the tumour and especially Level I LNs (34) .

There are challenges unique to SLNB in HNSCC including tongue cancers. It is a multidisciplinary technique and like all new techniques requires a learning curve and this is made more difficult as it requires all three stages, the preoperative lymphoscintigraphy, the intra-operative identification of the cervical SLN and the pathological assessment of the SLN, to be proficient before any results can be accurately assessed.

From the surgical view point, most cervical LNs are small even if they contain metastasis. Studies have shown that approximately 33% to 58% of occult metastases are smaller than 3 mm

and in 25% of patients, LNs smaller 3 mm were the only metastatic nodes detected in the neck. Intra-operative identification of these small nodes is a technique challenge, especially when the SLN is located within a group of nodes or embedded in fat tissue, in the complex anatomical region of the head and neck. This is complicated by the fact that lymphatic drainage patterns from head and neck sites are not as predictable as those observed in the extremities and multiple SLNs in multiple LN basins are common in SCC of the head and neck.

Factors in failures of SLNB were discussed by Hornstra et al. It is noted that patients with a negative pre-operative lymphoscintigraphy were found more likely to have an unsuccessful SNB procedure and notably, anterior tongue tumours or FOM tumours were also found to have a higher likelihood of SLNB failure due to a ‘‘shine through’’ phenomenon. This occurs due to the proximity of the sentinel node to the injection site. Patients with clinically palpable LNs or advanced primary tumors are independent factors associated with an unsuccessful SLNB. The presence of nodal involvement may lead to distortion of the normal lymphatic architecture by the tumour, blocking the normal drainage to the SLN and forcing an alternative drainage pathway, leading to inaccurate identification of the sentinel node or to complete failure of identification.

Sentinel lymph node biopsy (SLNB) has become an accepted diagnostic tool for determining whether metastasis are present in the first echelon of draining lymph nodes in melanoma and breast cancer patients. Sentinel lymph node biopsy has allowed these patients to avoid surgical morbidity while being accurately upstaged for their malignancies. The role of SLNB in the management of HNSCC patients is currently undefined and in the process of systematic validation. If this technique could be successfully applied to HNSCC, it would represent a significant advancement in staging these patients’ disease (35) .

6d. Controversies and issues regarding treatment options in Squamous Cell Carcinoma of Tongue

Treatment of squamous cell carcinoma of the tongue generally follows the same regime as any SCC of the Head and Neck. The biologically aggressive characteristic of oral SCC is well characterized by its ability to metastasize to cervical lymph nodes. The 5-year survival rate can decrease below 20% when cervical metastasis is present (36) . In management of SCC of the tongue, it is essential to note that in addition to the treatment of the primary tongue lesion, management of the involved cervical LNs also represents an important component of the therapeutic strategy. In the 1970s and still practiced in some parts of the world, the treatment regimen for oral SCC patients was to use a "wait-and-watch" policy for negative neck and to perform a form of ND only for a positive neck. After implementing the wait-and-watch policy, it was observed that some patients later presented with advanced unresectable lesions in the neck. Elective neck dissection (END) was accepted and incorporated into the treatment regime for clinically "negative" neck (cN0). Despite the advent of new imaging modalities like CT, MRI and recently PET-CT, due to the lack of any other precise non-invasive methods to predict cervical metastasis, elective neck dissection is still the recommended approach to patients with clinically negative neck (cN0) when the probability of occult metastasis is estimated to be in excess of 20% (37) .

Debate regarding the efficacy of END existed for more than four decades. The surgical technique of neck dissections has evolved from radical neck dissection (RND) to modified or functional neck dissection and then to selective neck dissection (SND). Selective neck dissection appears to

be effective as a staging procedure for cases with no clinical evidence of cervical nodal disease (cN0) and possibly also for cases with microscopic disease. Although this notion proposes an interesting possibility, until recently it is only supported by the results of retrospective studies including highly selected groups of patients. The purpose of SND is to selectively remove the lymphatic groups at the highest risk for metastasis and to decrease morbidity by preserving the 3 crucial structures namely, the sternocleidomastoid muscle, internal jugular vein, and the spinal accessory nerve, which is routinely dissected in RND.

As discussed earlier in the section under the role of neck dissection in HNSCC, radical neck dissection seemed to be unacceptable for patients with negative neck because of the poor quality of life due to the morbidities associated with it, such as shoulder disability, neck pain and long-term adverse disfiguring cosmetic effects. Recently, improved experience from longer follow-up data synergized with better knowledge of defined lymphatic drainage patterns in oral SCC prompted many head and neck surgeons to consider SND as the standard approach to clinically negative neck (cN0) (38) . The main advantage of SND is that it removes only the Level I, II and II LNs (the nodes at the greatest risk for metastasis) to decrease morbidity and post-operative dysfunction while the oncologic outcome remains unchanged. Review of the literature suggests that SND can provide valuable pathologic information for staging and further adjuvant treatment. However, the effectiveness of SND, especially compared with RND, has been controversial. Many published literature regarding the efficacy of supraomyohyoid neck dissection (SOHND) had shown disadvantages.

A major prospective trial by the Brazilian Head and Neck Cancer Study Group looked at the role of SOHND versus modified RND in the management of oral SCC (37) . In this study, all LNs suspected of metastasis in the SOHND group were submitted to frozen section, and the operations were converted to modified RND when neck nodes were found to be positive. The occult metastatic rate recurrence rates in the SOHND group were not significantly different from the RND group, therefore they conclude that SOHND seems to be as effective as RND in providing valuable staging information for patients with oral SCC and negative neck. For patients with nodal disease, postoperative radiotherapy did not significantly decrease recurrence rate in either the SOHND or RND groups. This suggests that such recurrences were related more to the aggressive biologic characteristics of oral SCC than the operative technique. In patients with nodal disease, consistent with other literature, SOHND alone in patients with positive neck was inadequate therapy for regional control without post-operative radiotherapy. The Brazilian Head and Neck Cancer Study Group concluded that the recurrence and survival rates were similar in both groups and SOHND can be recommended as standard elective treatment for patients with T2–T4 oral squamous cell carcinomas.

More recently, the questions are pertaining to the need to remove level IV LNs as skip metastasis is a known phenomenon (39) . The benefit for extension of SOHND to level IV nodes has been controversial so far. Current literature advocates routinely extending SOHND to level IV nodes in patients with SCC of the tongue and floor of the mouth because the procedure does not significantly increase operating time and adds little morbidity to the operation (40) . This study suggests that after careful examination of suspected level III lymph nodes by frozen section, extension to level IV nodes would be justified if they are found to be positive (41) .

7. Pathogenesis of a lymph node metastasis

Tumor cell metastasis to regional lymph nodes often marks the first step in tumor cell progression. The presence of metastatic cells in regional lymph nodes is the most important prognostic factor for malignant tumors of epithelial origin and along with extracapsular spread (ECS) of the cervical lymph nodes, they are the most important prognostic factors in patients with squamous cell carcinoma (SCC) of the tongue (24, 42-44) . In general, carcinomas metastasize through the lymphatic system more often than sarcomas, this may be explained by the fact that sarcomas expresses low levels of lymphatic stimulators and due to the presence of endogenous lymphangiogenic inhibitors. The lymphatic metastatic cascade is a series of complex interrelated steps and processes. It starts as the tumor grows and enlarges; cytokines are secreted to promote lymphangiogenesis. New lymphatics may grow, some within the tumor, but in the majority of carcinomas, they propagate towards the tumor (45) . As malignant cells invade the extracellular matrix, they enter the lymphatics lumen. They can move singly or in clusters to the sentinel or the first echelon of regional lymph nodes. This subset of tumor cells invades the cortex of the lymph node and proliferates and metastasizes to other lymph nodes. Traditionally viewed as a passive process, it has been suggested, but remains undetermined that the passage of tumors cells through the lymphatic channels is guided by mechanical forces. The interstitial fluid and hydrostatic pressure differential forces the cells and fluid towards the peri-tumor lymphatics (46) ; it is also guided by expression of adhesion receptors and the mechanism of chemokinesis (47) . Both processes are probably important and necessary. As the tumor grows, the interstitium swells and opens the intercellular clefts, facilitating the passage of fluid and cells into the lymphatic drainage away from the tumor with retrograde flow prevented by valves. These structural properties such as the lack of basement membrane in terminal lymphatics, presence of

the intercellular clefts. In addition, the traumatic serum environment in the blood stream, which are often toxic to the tumor cells, may account for the observation that in many solid tumors, lymphatic metastasis precedes metastasis via the vascular system.

Angiogenesis and lymphangiogenesis

Mammals possess two vascular systems, the blood vasculature and the lymphatic systems.

The lymphatic system consist of lymphoid organs such as the thymus, lymph nodes, the bone marrow, the spleen and specialized cells e.g. Peyer's patches connected by conduits called lymphatic vessels that are lined by single layer of lymphatic endothelial cells. The lymphatic conducting system carries the lymph and consists of tubular vessels that include the lymph capillaries, the lymph vessels, and the right and left thoracic ducts. The blood does not directly come in contact with the parenchymal cells and tissues in the body, but constituents of the blood first exit the microvascular exchange vessels to become interstitial fluid, which comes into contact with the parenchymal cells. Lymph is formed when interstitial fluid enters the initial lymphatic vessels of the lymphatic system. The lymph is then moved along the lymphatic vessel network by either intrinsic contractions of the lymphatic vessels or by extrinsic compression of the lymphatic vessels via external tissue forces e.g. the contractions of skeletal muscles.

The lymphatic system has several interrelated functions. It is responsible for the removal of interstitial fluid from tissues; it absorbs and transports fatty acids and fats as chyle to the circulatory system; it transports immune cells to and from the lymph nodes into tissues and

marrow. The lymph transports antigen-presenting cells (APCs), such as dendritic cells, to the lymph nodes where an immune response is stimulated. The lymph also carries lymphocytes from the efferent lymphatics exiting the lymph nodes. Lymphangiogenesis is the development and proliferation of new lymphatics from host vessels.

Blood vasculature consist of blood endothelial cells, basal membrane, smooth muscles cells and pericytes organizing into capillaries, venules, veins , arterioles and arteries in a circular manner, driven by the heart. It is the main system for transport of oxygen, carbon dioxide, nutrients, metabolic waste products, hormones and cells of the immune system and many factors around the body.

Angiogenesis is the generation of endothelial cells from existing blood vessels. If a cancerous tumor is larger than 1–2 mm in size, then angiogenesis is needed to prevent tumor cell apoptosis. Both systems, though distinctly different, have been shown with little doubt to be both functionally and morphologically related in many aspects. Tumor angiogenesis can occur in many ways and involve many complex processes and regulatory factors e.g. anti-angiogenic, pro-angiogenic factors. These include sprouting angiogenesis, intussusceptive angiogenesis, vasculogenesis, vasculogenic mimicry and vessel co-option to name a few (48). Many of the factors involved in this complex interplay include members of Vascular endothelial growth factor (VEGF) family, which is first identified in 1989. The VEGF family of growth factors now consists of six members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor), they are general activators of endothelial cell proliferation and mobility. As a group, it is

the most potent factor that increases permeability of the vessel wall, induces vasodilatation of the existing vessels, it increases the expression of matrix metalloproteinases and plasminogen activators for the degradation of the extracellular matrix and subsequently endothelial cell migration.

It is of no surprise and logical that the relationship between angiogenesis and lymphangiogenesis is extensively studied recently especially in cancer research. This is most pertinent in the biology of lymph node metastasis where the two systems literally lie side by side. There is now extensive direct evidence showing members of the VEGF family, namely VEGF-C,VEGF-D and recently VEGF-A (49), are not only important regulators of lymph vessel growth in vivo but also enhance lymphatic metastasis (45, 50-52) .

This is significant because there is now evidence that tumor can activate both lymph node lymphangiogenesis and angiogenesis before they metastasize which translates into logical efforts in largely neglected field of anti-cancer research targeting pathways of tumor lymphangiogenesis. In fact, there are encouraging results from therapeutic targeting of the VEGF receptor 3(VEGFR-3) pathway, which is the main activating pathway of which VEGF-C, VEGF-D are its ligands. Endostatin, 20-kDa C-terminal fragment of collagen XVIII, which is associated with down regulation of VEGF-A and VEGF-D, inhibited growth and lymph node metastasis of Squamous cell carcinoma (SCC) in xenograft models and demonstrated reduced lymphatic and vascular density (53) . Antibody to VEGF-D inhibits lymphangiogenesis and LN metastasis in a VEGF-D-dependent mouse tumor model (50) . A combination of both anti-

VEGFR-2 and VDGFR-3 blocking antibodies was shown to be more efficient in murine xenograft models to reduce overall tumor burden in the lymph nodes and distant metastases, than each alone (54) .

More than 25 anti-angiogenic drugs are in clinical trials. The first anti-angiogenic agent, bevacizumab (Avastin™), a monoclonal antibody to VEGF-A, was approved by FDA in 2004. It has proven clinical benefits in treatment of metastatic colorectal cancer when the drug was added to standard chemotherapy and also has recent positive results in metastatic breast cancer and lung cancer. There are more than 30 ongoing trials including Phase III clinical trials in advanced or metastatic renal cell carcinoma, pancreatic cancer, and ovarian cancer for Avastin™ (55).

Other molecular pathways that harbor potential targets which have varying degrees of anti-angiogenesis and anti-lymphangiogenesis are ligands such as hepatocyte growth factor (HGF), receptors like Neuropilin 2, PDGFRα/β and others such as Angiostatin, interferon α/β and platelet-factor 4 (56) . Two other antiangiogenic drugs, Sorafenib (Nexavar™, Bayer) and Sunitinib (Sutent™, Pfizer), have also been approved by the FDA. They target multiple receptor tyrosine kinases, including VEGF receptors and platelet-derived growth factor (PDGF) receptors (57) . Sorafenib and Sunitinib have been beneficial in the treatment of metastatic renal-cell cancer when used alone (58, 59) . Sorafenib monotherapy is also active in the liver and was recently approved by the FDA for treatment of hepatocellular carcinoma.

8. High endothelial venules (HEV) and its role

8a. Morphological Features and Functions

Lymph nodes are central to the pathogenesis of metastasis and the concept of sentinel LNs as the first lymphatic basin has been studied extensively in the latter half of the twentieth century. The concept that the pathological status of this special group of LNs is representative of the rest of the lymphatic basin and distant organs is now established in melanoma and carcinoma of the breast and benefited patients significantly as it has decreased surgical morbidity. If the first echelon group of lymph nodes is where the action lies, then the sentinel node is the gateway to this party. Blood vessels and lymphatic vessels maintain homeostasis in lymph nodes. High endothelial venules are specialized post-capillary venules found in lymphoid tissues, located mainly in the T-call zones such as the para-cortical areas of LNs. They are distinct from ordinary venules, morphologically and functionally, and besides the vascular endothelial cells (ECs) of the blood-brain barrier, the ECs of HEV are the next most well studied. Each HEV has a prominent peri-vascular sheath; a thick basal lamina and the layer of ECs are tall and plump in appearance. (Illustration 2) There are adhesion molecules common to all venules e.g. CD31, Intercellular adhesion molecule 2(ICAM-2), some particular molecules e.g. glycosylated and sulphated forms of sialomucins (also known as peripheral node addressins, PNADs), glycosylation-dependent cell-adhesion molecule 1(GLYCAM1), CD34, endomucin, podocalyxin and enodglycan. There are some lymphoid chemokines e.g. Chemokine ligand 19(CCL19), CCL21, CXC12 and CXCL13 are only highly expressed in HEV of peripheral LNs but not in other venules.

8b. High endothelial venules and its markers

High endothelial venules are characterized and studied extensively in the field of immunology. The evidence suggests that HEV has a central role in lymphocyte trafficking to LNs, allows the entrance of native L-selectin ^{high} cells in to the LN parenchyma and this is in large part mediated by chemokines produced in and around HEV such as the PNADs. There is a synchrony between HEV and lymphatic vessels revealed by immunization studies. LNs undergo remodeling with complex kinetics of lymph flow, cell content flow, blood flow, HEV gene expression and its morphological changes (60) .

8c. High endothelial venules' role in immunology and cancer

Recently, with animal models, it was shown that before the arrival of metastasis in the SLN, there are reorganizations of vasculature and lymphatic channels resulting in the SLN to become a functional blood vessel enriched organ. These prominent blood vessels are remodeled HEV. The extent of lymph sinus dilation correlated with the primary tumor weight and this is consistent with findings from other studies that, in contrast to angiogenesis, where flow only occurs after development of vessels, lymphangiogenesis can be induced by interstitial fluid channeling. The analogous role of HEV in immune function and cancer metastasis ends here. In inflammatory conditions, HEV's chief role in the traffic control of lymphocytes can be evident from the presence of lymphocytes in the dilated lymphatic sinuses; whereas in tumor-reactive lymphadenopathy, there are few cells, suggesting different processes, in which may be due to a change of HEV' role. There are also studies in murine models to suggest that the movement of tumor cells to LNs resembles the normal migration of dendritic cells during immune stimulation, resulting in the term "Tumor cell trafficking" (61) . This observation coupled with the

knowledge of the intimate relationship between HEV and lymphatic vessels in LNs leads to the logical hypothesis that HEV might provide the shortcut or a bypass route connecting the vascular and lymphatic system at the level of the SLN. This is in contrast to the Halstedian philosophy that an enbloc resection of the primary cancer and its regional lymphatic basin will achieve cure as its assumption is that tumor cells follow a stepwise pathway, from the primary tumor to the regional lymph nodes and to the next echelon and then to the systematic circulation through distal lymphaticovenous connections such as the thoracic duct. We know that this orderly fashion is not all true as from clinical follow-up studies, about 20% of women with node-negative breast cancer go on to develop distant metastases (62) . This is consistent with the analysis of sentinel lymphadenectomies suggesting that 20% of systemic metastases are derived from cancer cells that bypass this orderly lymphatic route (63) . The HEV providing the vehicle of this shortcut route for the cancer cells, might account for a subset of these patients.

In addition to this hypothetical role of providing the physical shortcut route in the LNs, the HEV evident from the presence of red blood cells within and significant increase size of the lumen in the presence of a tumor, hints that it presumably functions like a blood vessel in anticipation to supply the needs for an accelerating growth of a soon-to-arrive tumor deposit. This may explain in part that the tumor in nodes are frequently larger and faster growing than the primary itself like in the cases of nasopharyngeal carcinomas. This phenomenon is also consistent with the better chemotherapy and or radiotherapy responses in metastatic lesions of the breast and head and neck cancers, in part due to the lower proportion of hypoxic cancers cells and the increased delivery of the chemotherapeutic agents as a result of a better blood supply.

Remodeled HEV in the LN is shown to be capable of being further integrated into tumor vasculature after the secondary tumor nest is established in the LN (5, 64). This is the process of vessel co-option and this form or tumor angiogenesis is reported as an important component of tumor vasculature development in hepatocellular carcinoma (48).

9. Aim and Hypothesis of the Study

Specific aim:

We aim to confirm the morphological and functional alterations of high endothelial venules (**HEVs**) in the regional lymph nodes of the patients with carcinomas of the tongue and correlate these findings with clinical outcome.

Hypothesis:

Primary tongue cancer can induce vascular rearrangement in regional lymph node before metastasis to facilitate the growth of secondary cancer in the lymph node, resulting shorter patients' survival. The key blood vessels involved are high endothelial venules, with its function shifting from immune response to blood flow carrier. This transformation of HEV in the presence of cancer is a spectrum and these morphological features of altered HEV will correlate with clinical outcome of patients, establishing its pivotal role in the pathogenesis of metastasis. This association will serve high endothelial venules as a novel prognostic marker and a potential candidate for therapeutic targeting.

10. Patients, Materials and Methods

10a. Patients

This study is based on 175 consecutive patients with squamous cell carcinoma of the head and neck who have undergone primary surgical treatment at the Head and Neck Unit of the Department of General Surgery, Singapore General Hospital and the Department of Surgical Oncology, National Cancer Center, Singapore, from January 2001 through to December 2005. A retrospective review of these patients' pathological and clinical data including follow-up information was obtained from the Department of General Surgery Head and Neck database. For the purpose for this study, our inclusion criteria includes all surgically treated patients with histologically proven SCC tongue with any form of neck dissection and with a minimum of 2-year follow up period. Exculsion criteria includes all patients with non-SCC tongue cancers, patients with non-tongue SCC cancers of the Head and Neck, patients with any previously radiated or treated necks, patients with a second primary cancer and patients without a minimum of a 2 year follow up period, patients without surgery and/or a neck dissection as part of their primary treament.

All patients included had radical excision of the primary tongue lesion and a neck dissection according to our departmental protocol. In both of the cases and control group, all had radical enbloc excision of the primary tongue lesion with or without resection of adjacent structures with either a unilateral or a bilateral, supraomohyoid neck dissection or a modified radical neck dissection. Tumours were classified according to the AJCC TNM Staging Classification (22) , based on a pre-operative clinical evaulation and this determined the type of neck dissection

performed by the primary surgeon. Intra-operative frozen sections were performed for margins of the tongue lesion and further resection of the tongue was performed in event that the surgical margins were invovled.

Upon discharge from the hospital, they were followed up at regular intervals of monthly, 3-monthly, 6-monthly and yearly at a progessive rate according to individual surgeon's preference. Inpatient and outpatient clincal data, pathological, radiological and operative records were retrieved from a central medical records office and from the department of pathology. These information are retrospectively reviewed and entered into our departmental head and neck database.

10b, Immunohistochemistry

We conducted a retrospective review and analysis of the primary SCC tongue lesion and their respective lymph node tissues in the neck dissection specimen from the 65 cases of tongue cancer patients who had undergone radical surgery with sufficient clinical follow-up information. The lymph node tissues in each case are separated into 5 levels according to their anatomical locations.

Patient suitability and selection will be based on our inclusion and exclusion criteria. The tumor and the neck dissection paraffin tissue blocks from the selected patients are retrieved from the Department of Pathology, Singapore General Hospital

An independent head and neck pathologist(SK Hwang) reviewed the histological specimens and selected the 3 largest lymph node in both groups for staining of the HEV. The largest 3 lymph nodes were chosen as it is shown that size of a lymph node correlates with the degree of tumor lymphadenopathy and in the cases of the non-metastatic neck, the degree of reactive lymphadenopathy.

Optimisation of the immunohistochemical staining protocol and subsequent staining of the HEV using the MECA-79 antibody ,which is specific for HEV, was performed. (Table 3)

10c. Computer assisted Image Analysis

This was carried out in collaboration with the researchers at Van Andel Research Institute (VARI, Grand Rapids Michigan, U.S.A) and our institution. The digital capture of the HEV images and the quantitative image analyses are performed at Van Andel Research Institute (VARI) using the slide scanner(ScanScope, Aperio T3; ScanScope Console v. 9.0. Aperio Technologies, Vista, CA U.S.A) utilising an illuminator(Fiber-Lite DC-950, Dolan Jenner, Boxborough, MA, U.S.A).

We analyzed 3, 5 or 10 snapshots of largest 3 lymph nodes of 10x magnification, depending on the size of the LN. The total number of HEV, abnormal HEV, defined as having a lumen area of 80 square microns, and the HEV with presence of red blood cells were counted with the aid of imaging software (J image with cell counter plug-in) An average value for each of these

parameter were taken for every LN. These 3 values were co-related to the size of its respective LN and the patient's clinical data, namely, the overall survival, disease free interval and recurrence rate.

Definitions of the HEVs's parameters and ratios

This is represented by the following alphabets **A**, **B** and **C** for the ease of discussion in the rest of the results and discussion sections.

- no. of all HEV : **A**
- no. of dilated HEV (defined as lumen size more than 80square micron) : **B**
- no. of dilated HEV with red blood cells (rbcs) inside its lumen : **C**

10d. Statistical Analysis

Correlating with the clinical information from our prospective Head and Neck Cancer database, we analyzed the 3 parameters (**A**, **B**, **C**) and the 3 ratios (**B/A**, **C/B**, **C/A**) with respect to several clinical parameters, namely Disease Free Interval (DFI) and overall survival in the 2 groups(Cases vs. Controls) and as a whole cohort as well. We used Cox's Proportional Hazard Model for analysis.

For non-parametric data analysis, a non-parametric tests, e.g. Wilcoxon-rank-sum test and Kruskal-Wallis test, were performed in appropriate situations. E.g. to test the difference on different HEV parameters as well as the 3 ratios between control and case.

11. Results

There are 76 patients with histologically proven squamous cell carcinoma of the tongue and their histology were reviewed. This group of 76 patients are stratified according the presence or absence of pathologically proven lymph node metastases. Out of this SCC tongue group of 76 patients, 65 patients were included and 11 patients was excluded because of either the unavailability of the their tissue paraffin blocks and/or the lack of sufficient clinical and follow-up data.

The patients in the group with primary SCC tongue and absence of pathologically proven lymph node metastases in their neck dissection specimen are designated as "cases" and patients in the other group with primary SCC tongue and presence of pathologically proven lymph node metastases in the neck dissection specimens, they are designated as "controls".

As detailed in the Material and Method's section, we performed image analysis looking at the number of HEVs, the number of dilated HEVs (defined as lumen size more than 80 square micron) and lastly no. of dilated HEV with red blood cells inside its lumen in the 3 largest LN of each patient and achieved an average number for each patient based on a HPF (10 X 10) magnification field. This is represented by the following alphabets **A**, **B** and **C** for the ease of discussion in the rest of the results and discussion sections. (Illustration 3)

- no. of all HEVs : **A**
- no. of dilated HEVs (defined as lumen size more than 80square micron) : **B**
- no. of dilated HEVs with red blood cells (rbcs) inside its lumen : **C**

In addition to these three parameters, we also analyzed the **ratios** of these abnormal HEVs (**B** and **C**) with respect to the total number of HEVs (**A**). This is summarized below as

- Percentage of dilated HEVs with respect to total no. of HEVs i.e. Ratio of dilated HEVs to the total number of HEVs : **B/A**
- Percentage of dilated HEVs with rbcs within its lumen with respect to total no. of dilated all HEVs i.e. Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs : **C/B**
- Percentage of dilated HEVs with rbcs within its lumen with respect to total no. HEVs : **C/A**

10a. Summary of Results

Disease Free Interval analysis

The sample data suggested a statistically significant association of number of HEVs (**A**) to the disease free interval (DFI). This is statistically significant when controlling for the group (control vs. case) (p-value = 0.022). There is also statistical significance when we combined both group as one cohort (control + case) (p-value = 0.023). The sample data did not detect any significance in DFI between controls and cases (p-value = 0.472). (Table 4 Summary of results)

Overall survival analysis

The sample data suggested marginal significance in terms of overall survival time between controls and cases (p-value = 0.066). (Figure 8) There was no significance detected found in the total no. of HEVs (**A**); no. of dilated HEVs (**B**); no. of dilated HEVs with rbcs inside its lumen(**C**) or any ratios(**B/A**, **C/B**, **C/A**) with respect to the overall survival.

Table 4: Summary of results

HEV parameters	Clinical data	Relative Risk	p
Total no. of HEVs (A)	Overall Survival	1.024	0.471
	Disease Free Interval	1.051	**0.022**
Total no. of HEVs (A) and Disease Free Interval (as a Cohort)	Disease Free Interval	1.051	**0.023**
Dilated HEVs (B)	Overall Survival	1.071	0.476
	Disease Free Interval	1.034	0.594
HEV with rbcs within its lumen (C)	Overall Survival	1.116	0.345
	Disease Free Interval	1.044	0.584
Ratio of dilated HEVs to the total no. of HEVs(**B**/**A**)	Overall Survival	1.078	0.982
	Disease Free Interval	10.10	0.450
Ratio of dilated HEVs with rbcs to total no. HEV (**C**/**A**)	Overall Survival	3.624	0.737
	Disease Free Interval	4.67	0.643
Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs (**C**/**B**)	Overall Survival	17.884	0.171
	Disease Free Interval	5.458	0.208

10b. Main Results in Detail

We analyzed the clinical parameters of Disease Free Interval (DFI) and overall survival (OS) of the patients with respective to their groups (Cases versus Controls). The patients with no lymph node metastases in the neck dissection specimen are designated as CASES and patients with positive lymph node metastases are designated as CONTROLS. This is analyzed in an effort to establish or exclude whether the presence of metastasis in the regional cervical LNs is a factor in the HEV reorganization.

Statistical Analysis:

1.) Fit the 'disease free interval (DFI)' and 'overall survival (OS)'for two groups of patients (control vs. case)

Let $$X_1 = \begin{cases} \mathbf{0} & \mathbf{control} \\ \mathbf{1} & \mathbf{case} \end{cases}$$

Then the hazard function of an patient *j*, $\lambda_j(t)$, with certain X_1 value can be expressed as

$$\lambda_j(t) = \lambda_0(t) \cdot e^{\beta_1 x_1} \quad \text{or} \quad \lambda_j(t) = \lambda_0(t) \cdot \exp(\beta_1 x_1)$$

Where $\lambda_0(t)$ is the baseline hazard.

A total of 65 patients with 30 controls and 35 cases were included in the analysis to estimate the relative risk of disease free interval and overall survival associated with the group (control vs. case).

1.A) Overall Survival:

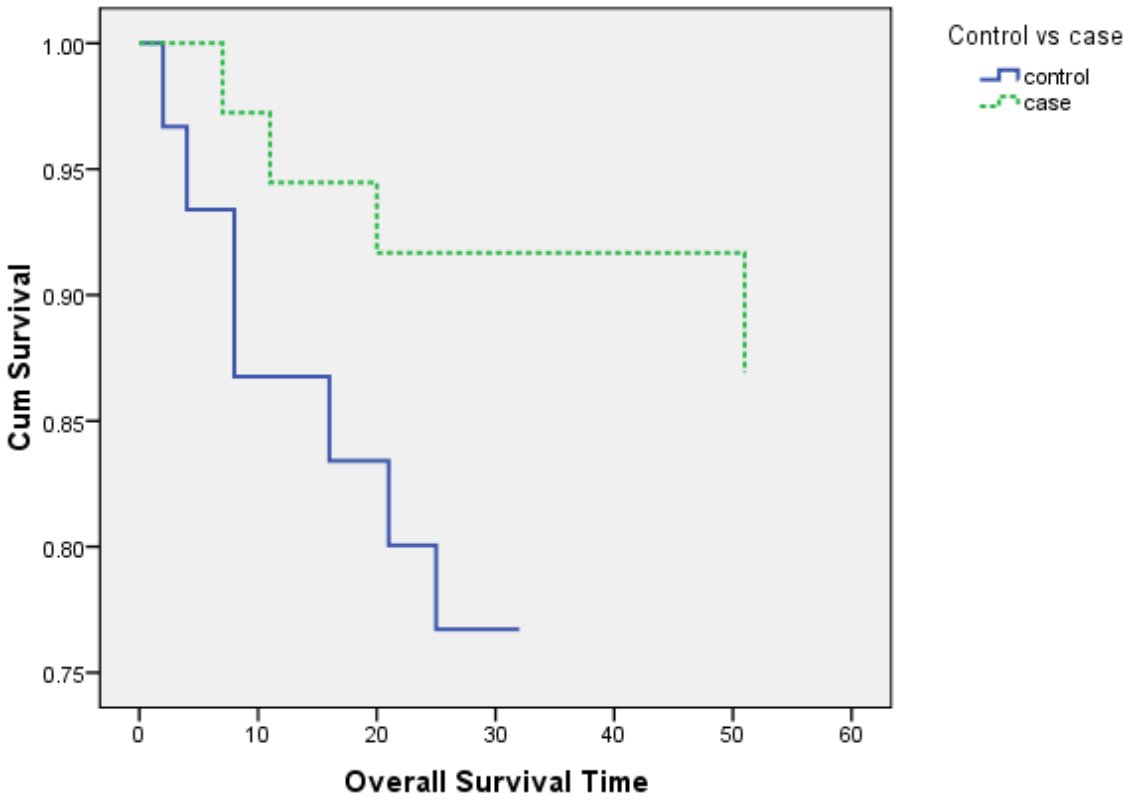
Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Group	-1.475	.802	3.383	1	.066	.229	.048	1.102

We compared the overall survival between cases and controls and analyzed the relative risk [exp (B)] in the above output. The risk of case group is estimated to be 0.229 times of the risk of control group based on the study sample (a 95% C.I. is 0.048 ~ 1.102). (Figure 8)

In another words, the risk of control group is $\frac{1}{0.229} = 4.367$ times higher. This equates that the patients with presence of metastases in their regional cervical LNs have a **4.367** higher chance of dying as compared to patients without metastasis in their regional LNs. This is marginally significant (p-value = 0.066).

Figure 4

Kaplan Meier Overall Survival curves for the two groups (Cases vs. Controls)



p-value = 0.066

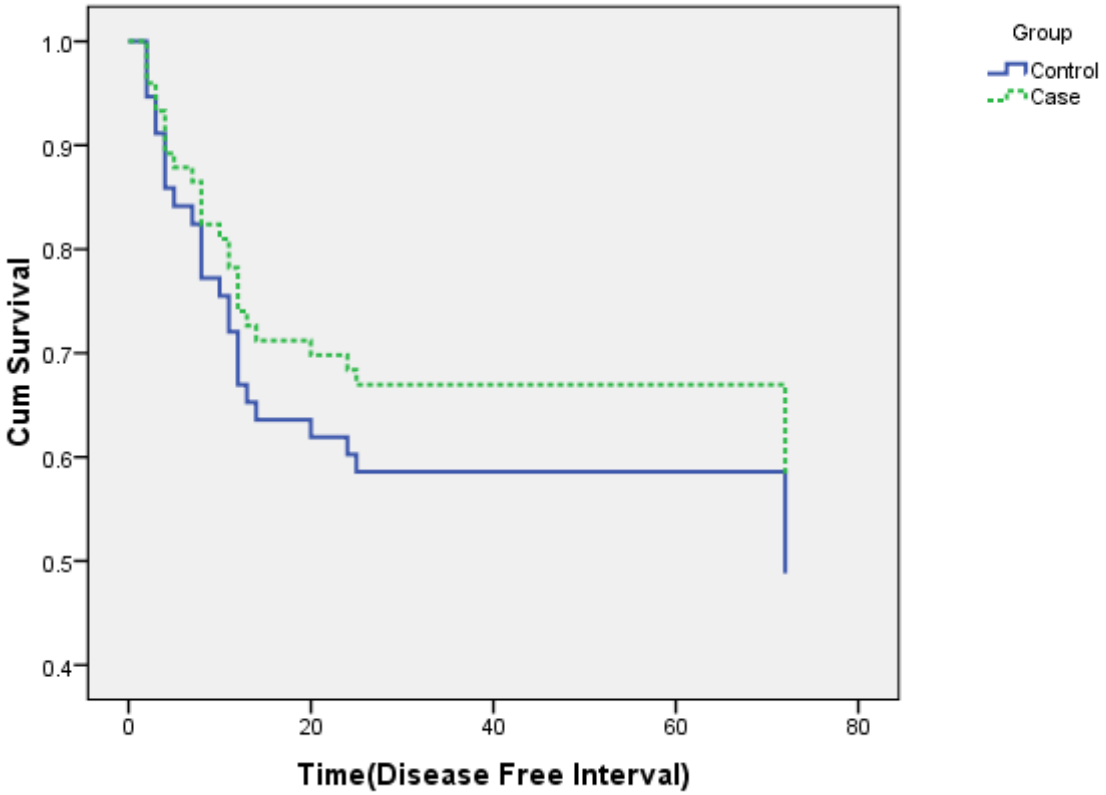
1.B) Disease free interval:

Similarly, we compared the disease free interval between control and case and looked at the relative risk [exp (B)] in the above output. The risk of case group is estimated to be 0.75 times of the risk of control group based on the sample (a 95% C.I. is 0.342 ~ 1.644). This implies that in patients without LN metastases, if recurrences occur, the DFI tend to be longer. However, this did not achieve is not statistical significance (p-value = 0.472) (Figure 9)

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Group	-.288	.400	.517	1	.472	.750	.342	1.644

Figure 5 : Disease free interval curves for the two groups (Cases vs. Controls)



2.) In the **second** part, we analyzed the DFI and OS for the 2 groups (cases vs. controls) with respect to the 3 HEVs parameters (**A**, **B**, **C**)

- Total no. of HEVs : **A**
- no. of dilated HEVs (defined as lumen size more than 80 square micron) : **B**
- no. of dilated HEVs with red blood cells (rbcs) inside its lumen : **C**

Fit the 'disease free interval (DFI)' and 'overall survival (OS)' for the two groups (control vs. case) with A, B and C values.

Let $X_1 = \begin{cases} 0 & \textbf{control} \\ 1 & \textbf{case} \end{cases}$ and X_2 be the values of HEV or Dilated HEV or RBC

Then the hazard function of a patient *j*, $\lambda_j(t)$, with certain X_1 and X_2 values can be expressed as

$$\lambda_j(t) = \lambda_0(t) \cdot e^{\beta_1 x_1 + \beta_2 x_2} \qquad \text{or} \qquad \lambda_j(t) = \lambda_0(t) \cdot \exp(\beta_1 x_1 + \beta_2 x_2)$$

Where $\lambda_0(t)$ is the baseline hazard.

2.A) Analysis of Overall Survival and HEVs parameters (A, B, C)

a). Association of **A** (total no. of HEV) and OS–

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Group	-.848	.630	1.813	1	.178	.428	.125	1.472
HEV(A)	.023	.032	.520	1	.471	1.024	.961	1.091

Variables in the Equation

To observe the association of **A** (total no. of HEV) on overall survival while controlling for the group (control vs. case), we looked at the relative risk [exp(B) – in purple circle] in the above output. While controlling for the group, the risk is estimated to be **1.024 times** when the HEV value increase by 1 based on the sample (a 95% C.I. is 0.961 ~ 1.091). However, the association of **A** (total no. of HEV) and overall survival is not significant while controlling for group (p-value = 0.955)

To further illustrate the meaning of the above output, we write up the hazard functions under certain group and HEV values:

$$\lambda_j(t) = \lambda_0(t) \cdot \exp(-1.477 x_1 + 0.002 x_2)$$

	Control or Case	Total no. of HEV(A)	Relative risk
Control & HEV = 0	0	0	1.00
Control & HEV = 1	0	1	1.024
Control & HEV = 2	0	2	$\mathbf{(1.024)^2}$
Case & HEV = 0	1	0	0.428
Case & HEV = 1	1	1	$\mathbf{0.428 \times 1.024}$
Case & HEV = 2	1	2	$\mathbf{0.428 \times (1.024)^2}$

b). Association of **B** ('no. of dilated HEV') and OS –

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group	-.904	.651	1.932	1	.165	.405	.113	1.449
Dilated HEVs(**B**)	.069	.097	.507	1	.476	1.071	.886	1.295

To observe the association of **B** ('no. of dilated HEV') on overall survival while controlling for the group (control vs. case), we can look at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be **1.071** times when the dilated HEV value increase by 1 based on the sample (a 95% C.I. is 0.886 ~ 1.295). However, the association of **B** ('no. of dilated HEV') and overall survival is not statistically significant while controlling for group (p-value = 0.476)

To further illustrate the meaning of the above output, we write up the hazard functions under certain group and **B** ('no. of dilated HEV') values:

$$\lambda_j(t) = \lambda_0(t) \cdot \exp(-1.531x_1 + 0.05x_2)$$

	Control (0) or Case (1)	Dilated HEV(**B**)	Relative risk
Control & **B** = 0	0	0	1.00
Control & **B** = 1	0	1	1.071
Control & **B** = 2	0	2	$\mathbf{(1.071)^2}$
Case & **B** = 0	1	0	0.405
Case & **B** = 1	1	1	$\mathbf{0.405 \times 1.071}$
Case & **B** = 2	1	2	$\mathbf{0.405 \times (1.071)^2}$

c). Association of **C** ('HEV with red blood cells within its lumen') and OS–

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Group	-.851	.632	1.813	1	.178	.427	.124	1.474
HEV with rbcs (**C**)	.110	.116	.893	1	.345	1.116	.889	1.401

To observe the association of **C** ('HEV with red blood cells within its lumen') on overall survival while controlling for the group (control vs. case), we can look at the relative risk [exp(B) – in purple circle] in the above output. While controlling for the group, the risk is estimated to be **1.116** times when the RBCs in HEV value increase by 1 based on the sample (a 95% C.I. is

0.889 ~ 1.401)). However, the association of **C** ('HEV with red blood cells within its lumen') and overall survival is not statically significant while controlling for group (p-value = 0.345)

2.B) Analysis of Disease Free Interval and HEVs parameters (A, B, C)

a). Association of the **A** (total no. of HEV) and DFI

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Group	-.312	.401	.604	1	.437	.732	.334	1.607
HEV (A)	.050	.022	5.215	1	.022	1.051	1.007	1.097

To observe the association of HEV on disease free interval while controlling for the group (control vs. case), we looked at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be **1.051** times when the HEV value increase by 1 based on the sample (a 95% C.I. is 1.007 ~ 1.097).

The association of **A** (total no. of HEV) and disease free interval is **significant** while controlling for the group (p-value = 0.022*).

b). Association of the number of **B** ('no. of dilated HEV') and DFI–

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group	-.319	.407	.616	1	.432	.727	.327	1.613
Dilated HEVs (B)	.033	.063	.284	1	.594	1.034	.915	1.169

To observe the association of **B** ('no. of dilated HEV') on disease free interval while controlling for the group (control vs. case), we looked at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be **1.034** times when the dilated HEV value increase by 1 based on the sample (a 95% C.I. is 0.915 ~ 1.169). However, the association on **B** ('no. of dilated HEV') and disease free interval is not significant while controlling for group (p-value = 0.594)

c). Association of **C** ('HEV with red blood cells within its lumen') and DFI –

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group	-.291	.401	.527	1	.468	.748	.341	1.640
HEV with rbcs (**C**)	.043	.078	.300	1	.584	1.044	.896	1.216

To observe the association of **C** ('HEV with red blood cells within its lumen') on disease free interval while controlling for the group (control vs. case), we looked at the relative risk [exp(B) –

in purple circle] in the above output. While controlling for the group, the risk is estimated to be **1.044** times when the RBCs in HEV value increase by 1 based on the sample (a 95% C.I. is 0.896 ~ 1.216). However, the association on **C** ('HEV with red blood cells within its lumen') and disease free interval is not statistically significant while controlling for group (p-value = 0.584)

3.) In the **third** part of our analysis, we further analyzed the DFI and OS for the 2 groups (cases vs. controls) with respect to the 3 ratios of HEVs parameters, as detailed below.

- Ratio of dilated HEVs to the total number of HEVs : **B/A**
- Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs : **C/A**
- Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs : **C/B**

We fit the 'disease free interval (DFI)' and 'overall survival (OS)' for the two groups (control vs. case) with ratios of HEV and Dilated HEV and RBC values

- Let $X_1 = \begin{cases} 0 & \textbf{control} \\ 1 & \textbf{case} \end{cases}$ and X_2 be the ratio values of **B/A** or **C/A** or **C/B**

Then the hazard function of an individual *j*, $\lambda_j(t)$, with certain X_1 and X_2 values can be expressed as

$$\lambda_j(t) = \lambda_0(t) \cdot e^{\beta_1 x_1 + \beta_2 x_2} \qquad \text{or} \qquad \lambda_j(t) = \lambda_0(t) \cdot \exp(\beta_1 x_1 + \beta_2 x_2)$$

Where $\lambda_0(t)$ is the baseline hazard.

3.A) Overall Survival and HEV ratios

a) Association of the ' Ratio of dilated HEVs to the total number of HEVs(**B/A**)' and Overall Survival

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
B/A	.075	3.257	.001	1	.982	1.078	.002	638.234

To observe the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.078 times when the ratio value increase by 1 based on the sample. However, the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') and overall survival is not significant (p-value = 0.982).

b) Association of the **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') and Overall Survival

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
C/A	1.288	3.833	.113	1	.737	3.624	.002	6634.427

To observe the association of **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') on overall survival, we can look at the relative risk, exp(B) in the above output. The risk is estimated to be 3.624 times when the ratio value increase by 1 based on the sample. However, the association of **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs) and overall survival is not significant (p-value = 0.737).

c) Association of the **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs')and Overall Survival

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
C/B	2.884	2.108	1.871	1	.171	17.884	.287	1114.678

To observe the association of the **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') on overall survival, we can look at the relative risk, exp(B) in the above output. The risk is estimated to be 17.884 times when the ratio value increase by 1 based on the sample. However, the association of **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') and overall survival is not significant (p-value = 0.171).

3.B) Disease Free Interval and HEV ratios:

a) Association of the **B/A** ('Ratio of dilated HEVs to the total number of HEVs') and Disease Free Interval

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group	-.263	.401	.430	1	.512	.769	.351	1.687
B/A	-2.314	3.061	.571	1	.450	.099	.000	39.890

To observe the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') on disease free interval while controlling for the group (control vs. case), we can look at the relative

risk, exp(B), in the above output. While controlling for the group, the risk is estimated to be 0.099 times when the ratio value increase by 1 based on the sample. In another word, the risk will be about 10 times ($\frac{1}{0.099} = 10.1$) higher when the ratio decreases by 1. However, the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') and disease free interval is not significant while controlling for the group (p-value = 0.450).

b) Association of the **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') and Disease Free Interval

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Group	-.287	.401	.513	1	.474	.751	.342	1.645
C/A	-1.541	3.328	.214	1	.643	.214	.000	145.830

To observe the association of **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') on disease free interval while controlling for the group (control vs. case), we can look at the relative risk, exp(B),in the above output. While controlling for the group, the risk is estimated to be 0.214 times when the ratio value increase by 1 based on the sample. In another words, the risk will be about 4.67 times ($\frac{1}{0.214} = 4.67$) higher when ration decrease by 1. However, the association of **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') and disease free interval is not significant while controlling for the group (p-value = 0.643).

c) Association of the **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs") and Disease Free Interval

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group	-.150	.416	.129	1	.719	.861	.381	1.947
C/B	1.697	1.348	1.585	1	.208	5.458	.389	76.616

To observe the association of the **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') on disease free interval while controlling for the group (control vs. case), we can look at the relative risk, exp(B), in the above output. While controlling for the group, the risk is estimated to be 5.458 times when the ratio value increase by 1 based on the sample. However, the association of **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') and disease free interval is not significant while controlling for the group (p-value = 0.208).

4.) In the **fourth** part, we analyzed the DFI and OS with respect to the 3 HEVs parameters (**A**, **B**, **C**) and the 3 Ratios **B/A**; **C/A** ; **C/B without consider the groups. In another words, we analysed all the patients as a single cohort.** We fit the 'disease free interval (DFI)' and 'overall survival (OS)' with the HEVs parameters or the ratios without considering the group.

Let X_1 be the values of **A** or **B** or **C** or **B/A** or **C/A** or **C/B**

Then the hazard function of an individual *j*, $\lambda_j(t)$, with certain X_1 value can be expressed as

$$\lambda_j(t) = \lambda_0(t) \cdot e^{\beta_1 x_1} \quad \text{or} \quad \lambda_j(t) = \lambda_0(t) \cdot \exp(\beta_1 x_1)$$

Where $\lambda_0(t)$ is the baseline hazard.

4.A) Overall Survival and HEV parameters as a cohort (i.e. without group effect)

a) Association of Total no. of HEVs (**A**) and OS(as a Cohort)

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
HEV (A)	-.003	.035	.006	1	.941	.997	.932	1.067

To observe the association of **A** (total no. of HEV) on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 0.997 times when the HEV value increase by 1 based on the sample (a 95% C.I. is 0.932 ~ 1.067). However, the association of **A** (total no. of HEV) and overall survival is not significant (p-value = 0.941)

b) Association of **B** ('no. of dilated HEV') and OS (as a Cohort)

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Dilated HEVs (B)	.004	.111	.001	1	.972	1.004	.808	1.247

To observe the association of **B** ('no. of dilated HEV') on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.004 times when the dilated HEV value increase by 1 based on the sample (a 95% C.I. is 0.808 ~ 1.247). However, the association of **B** ('no. of dilated HEV') and overall survival is not significant (p-value = 0.972)

c) Association of HEVs with red blood cells within its lumen (**C**) and OS (as a Cohort)

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
HEV with rbcs (**C**)	.008	.135	.003	1	.954	1.008	.774	1.313

To observe the association of **C** ('HEV with red blood cells within its lumen') on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be

1.008 times when the **C** ('HEV with red blood cells within its lumen') increase by 1 based on the sample (a 95% C.I. is 0.774 ~ 1.313). However, the association of **C** ('HEV with red blood cells within its lumen') and overall survival is not significant (p-value = 0.954)

d) Association of the **B/A** ('Ratio of dilated HEVs to the total number of HEVs') and Overall Survival(as a Cohort)

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
B/A	.168	3.656	.002	1	.963	1.183	.001	1532.264

To observe the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.183 times when the ratio value increase by 1 based on the sample. However, the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') and overall survival is not significant (p-value = 0.963).

e) Association of the **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') and Overall Survival (as a Cohort)

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
C/A	.160	4.923	.001	1	.974	1.174	.000	18189.501

To observe the association of **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') on overall survival, we can look at the relative risk, exp(B) in the above output. The risk is estimated to be 1.174 times when the ratio value increase by 1 based on the sample. However, the association of **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs')and overall survival is not significant (p-value = 0.974).

f) Association of the **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') and Overall Survival (as a Cohort)

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
C/B	.954	1.997	.228	1	.633	2.597	.052	130.178

To observe the association of **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') on overall survival, we can look at the relative risk, exp(B) in the above output. The risk is estimated to be 2.597 times when the ratio value increase by 1 based on the sample. However, the association of **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') and overall survival is not significant (p-value = 0.633).

4.B)) Disease Free Interval and HEV parameters as a cohort (i.e. without group effect)

Disease Free Interval:

a). Association of Total no. of HEVs (**A**) and **Disease Free Interval** (as a Cohort)

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
HEV (A)	.049	.022	5.189	1	.023	1.051	1.007	1.096

To observe the association of **A** (total no. of HEV) on disease free interval, we can look at the relative risk (exp(B) – in purple circle) in the above output. The risk is estimated to be 1.051 times when the **A** (total no. of HEV) value increase by 1 based on the sample (a 95% C.I. is 1.007 ~ 1.096). The association of **A** (total no. of HEV) and disease free interval is **significant** (p-value = 0.023*).

b) Association of Dilated HEVs (**B**) and DFI (as a Cohort)

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Dilated HEVs(**B**)	.025	.060	.175	1	.676	1.026	.911	1.154

To observe the association of **B** ('no. of dilated HEV') on disease free interval, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.026 times when the

dilated HEV value increase by 1 based on the sample (a 95% C.I. is 0.911 ~ 1.154). However, the association of **B** ('no. of dilated HEV') and disease free is not significant (p-value = 0.676)

c) Association of **C** ('HEV with red blood cells within its lumen') and DFI (as a Cohort)

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
RBCsinHEV	.041	.076	.289	1	.591	1.042	.897	1.210

To observe the association **C** ('HEV with red blood cells within its lumen') on disease free interval, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.042 times when the **C** ('HEV with red blood cells within its lumen') value increase by 1 based on the sample (a 95% C.I. is 0.897 ~ 1.21). However, the association of **C** ('HEV with red blood cells within its lumen') and disease free is not significant (p-value = 0.591)

d) Association of the **B/A** ('Ratio of dilated HEVs to the total number of HEVs') and DFI (as a Cohort)

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
B/A	-2.358	2.971	.630	1	.427	.095	.000	31.994

To observe the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') on disease free interval, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 0.095 times when the ratio value increase by 1 based on the sample. In another word, the risk will be about 10.5 times ($\mathbf{1/0.095 = 10.5}$) higher when ration decrease by 1. However, the association of **B/A** ('Ratio of dilated HEVs to the total number of HEVs') and disease free interval is not significant (p-value = 0.427).

e) Association of the **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') and DFI (as a Cohort)

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
C/A	-1.491	3.202	.217	1	.641	.225	.000	119.632

To observe the association of **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') on disease free interval, we can look at the relative risk, exp(B) in the above output. The risk is estimated to be 0.225 times when the ratio value increase by 1 based on the sample. In another word, the risk will be about 4.44 times ($\mathbf{1/0.225 = 4.44}$) higher when ration decrease by 1. However, the association of the **C/A** ('Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs') and disease free interval is not significant (p-value = 0.641).

f). Association of the **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') and DFI (as a Cohort)

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
C/B	1.831	1.306	1.966	1	.161	6.243	.482	80.789

To observe the association **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') on disease free interval, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 6.243 times when the ratio value increase by 1 based on the sample. . However, the association of the **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') and disease free interval is not significant (p-value = 0.161).

11c. Supplementary Data Analysis Results

In this final section, utilizing information from our prospective data collection, we further analyzed our tumor pathological data with respect to the primary tumor volume, the stage of the disease and grade of the tumor against the patient's overall survival and disease free interval.

In addition, we also looked into the relationship of the HEV parameters and the 3 ratios previously defined and analyzed them against the tumor pathological characteristics, namely the primary tumor volume, the stage of the disease and grade of the tumor.

Summary of the secondary analysis (Table 5)

Tumor Characteristics	**Clinical data**	**Relative Risk**	**p**
Tumor volume (TV)	Overall Survival	0.985	0.476
	Disease Free Interval	0.990	0.481
Stage (S)	Overall Survival	1.116	0.71
	Disease Free Interval	1.302	0.348
Grade(G)	Overall Survival	0.882	0.815
	Disease Free Interval	1.436	0.308
Since there are no statistical difference noted between the 2 groups, we now consider the 2 groups (Cases and Controls) as a cohort and repeat the analysis summarized below (i.e. without considering the group)			
Tumor volume (TV_c)	Overall Survival	0.994	0.765
	Disease Free Interval	0.994	0.648
Stage (S_c)	Overall Survival	1.364	0.237
	Disease Free Interval	1.209	0.255
Grade(G_c)	Overall Survival	1.121	0.822
	Disease Free Interval	1.493	0.221

Supplementary Analysis in detail

Using Cox's Proportional Hazard Model for Overall Survival / Disease Free Interval

(Tumor volume, stage, grade)

1.) Fit the 'overall survival (OS)' or 'disease free interval (DFI)' for the two groups (control vs. case) with tumor volume (or stage, or grade)

Let $X_1 = \begin{cases} 0 & \textbf{control} \\ 1 & \textbf{case} \end{cases}$ and X_2 be the values of tumor or stage or grade

Then the hazard function of an individual *j*, $\lambda_j(t)$, with certain X_1 and X_2 values can be expressed as

$$\lambda_j(t) = \lambda_0(t) \cdot e^{\beta_1 x_1 + \beta_2 x_2} \qquad \text{or} \qquad \lambda_j(t) = \lambda_0(t) \cdot \exp(\beta_1 x_1 + \beta_2 x_2)$$

Where $\lambda_0(t)$ is the baseline hazard.

1.A) Overall Survival:

a). Tumor volume –

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
group	-.955	.649	2.163	1	.141	.385	.108	1.374
Tumor volume	-.015	.022	.508	1	.476	.985	.944	1.027

To observe the association of tumor volume on overall survival while controlling for the group (control vs. case), we can look at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be 0.985 times when the tumor volume

increase by 1 unit based on the sample. However, the association of Tumor volume (TV) and overall survival is not significant while controlling for group (p-value = 0.476).

Please note that, in both control and case groups, the tumor volumes among the events (death) relatively small comparing to that among the non-death.

b). Stage (S) –

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
group	-.607	.993	.374	1	.541	.545	.078	3.817
Stage	.109	.412	.071	1	.790	1.116	.498	2.501

To observe the association of 'stage' on overall survival while controlling for the group (control vs. case), we can look at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be 1.116 times when the advance to next stage based on the sample (a 95% C.I. is 0.498 ~ 2.501). However, the association on stage and overall survival is not significant while controlling for group (p-value = 0.79)

c). Grade (G) –

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
group	-.862	.663	1.692	1	.193	.422	.115	1.548
Grade	-.125	.536	.055	1	.815	.882	.309	2.521

To observe the association of 'grade' on overall survival while controlling for the group (control vs. case), we can look at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be 0.882 times when advance to next grade based on the sample (a 95% C.I. is 0.309 ~ 2.521). However, the association on grade and overall survival is not significant while controlling for group (p-value = 0.815)

1.B) Disease Free Interval (DFI):

a). Tumor(TV)–

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
group	-.383	.418	.839	1	.360	.682	.301	1.547
tumor_V	-.010	.014	.496	1	.481	.990	.963	1.018

To observe the association of tumor volume on disease free interval while controlling for the group (control vs. case), we can look at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be 0.990 times when the tumor volume increase by 1 unit based on the sample. However, the association on HEV and disease free interval is not significant while controlling for group (p-value = 0.481).

b). Stage (S) –

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
group	.222	.672	.109	1	.741	1.249	.335	4.656

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
group	.222	.672	.109	1	.741	1.249	.335	4.656
stage	.264	.282	.880	1	.348	1.302	.750	2.261

To observe the association of 'stage' on disease free interval while controlling for the group (control vs. case), we can look at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be 1.302 times when the advance to next stage based on the sample (a 95% C.I. is 0.750 ~ 2.261). However, the association on stage and disease free interval is not significant while controlling for group (p-value = 0.348)

c). Grade –

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
group	-.120	.436	.075	1	.784	.887	.377	2.085
Grade(G)	.362	.355	1.038	1	.308	1.436	.716	2.881

To observe the association of 'grade' on disease free interval while controlling for the group (control vs. case), we can look at the relative risk (exp(B) – in purple circle) in the above output. While controlling for the group, the risk is estimated to be 1.436 times when advance to next grade based on the sample (a 95% C.I. is 0.716 ~ 2.881). However, the association on grade and disease free interval is not significant while controlling for group (p-value = 0.308)

2.) Fit the 'overall survival (OS)' or 'disease free interval (DFI) with tumor volume (or stage, or grade) without considering the group (since there is no significance found on the risk between two groups, we may combine the two groups into one)

Let $\boldsymbol{X_1}$ be the values of tumor volume or stage or grade

Then the hazard function of an individual *j*, $\boldsymbol{\lambda_j(t)}$, with certain $\boldsymbol{X_1}$ value can be expressed as

$$\boldsymbol{\lambda_j(t) = \lambda_0(t) \cdot e^{\beta_1 x_1}} \quad \text{or} \quad \boldsymbol{\lambda_j(t) = \lambda_0(t) \cdot \exp(\beta_1 x_1)}$$

Where $\boldsymbol{\lambda_0(t)}$ is the baseline hazard.

In this section, we consider all patients as a single cohort and performed the analysis again.

(**Subscript c** signifying the analysis of the various parameters with regards to all patients considered as a single cohort)

2.A) Overall Survival:

a). Tumor Volume (TV_c) –

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
TVc	-.006	.020	.089	1	.765	.994	.955	1.034

To observe the association of tumor volume on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 0.994 times when the tumor volume increase by 1 unit based on the sample (a 95% C.I. is 0.955 ~ 1.034). However, the association on tumor volume and overall survival is not significant (p-value = 0.765)

b). Stage (S_c) –

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
S_c	.311	.263	1.397	1	.237	1.364	.815	2.284

To observe the association of stage on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.364 times when advance to next stage based on the sample (a 95% C.I. is 0.815 ~ 2.284). However, the association on stage and overall survival is not significant (p-value = 0.237)

c). Grade (G_c)–

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
G_c	.114	.508	.051	1	.822	1.121	.414	3.037

To observe the association of grade on overall survival, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.121 times when advance to next grade based on the sample (a 95% C.I. is 0.414 ~ 3.037). However, the association on grade and overall survival is not significant (p-value = 0.822)

2.B) Disease Free Interval:

a). Tumor Volume(TV_c) –

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
TV_c	-.006	.013	.209	1	.648	.994	.968	1.020

To observe the association of tumor volume on disease free interval, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 0.994 times when the tumor volume increase by 1 unit based on the sample (a 95% C.I. is 0.968 ~ 1.020). However, the association on tumor volume and disease free interval is not significant (p-value = 0.648)

b). Stage (S_c) –

Variables in the Equation								
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
S_c	.190	.167	1.294	1	.255	1.209	.872	1.676

To observe the association of stage on disease free interval, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.209 times when advance to next stage based on the sample (a 95% C.I. is 0.872 ~ 1.676). However, the association on stage and disease free interval is not significant (p-value = 0.255)

c). Grade(G_c)–

Variables in the Equation								
							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
grade	.401	.328	1.496	1	.221	1.493	.786	2.838

To observe the association of grade on disease free interval, we can look at the relative risk, exp(B), in the above output. The risk is estimated to be 1.493 times when advance to next grade based on the sample (a 95% C.I. is 0.786 ~ 2,838). However, the association on grade and disease free interval is not significant (p-value = 0.221).

In the **second** part of the supplementary analysis, we compared the HEV parameters in the 2 groups as well as the HEV parameters to the tumor characteristics i.e. tumor stage and its grade. Tumor volume is excluded as there are it is a continuous variable.

3 A. Control vs. Case – differences on average HEV and 3 ratios:

A non-parametric test, Wilcoxon-rank-sum test, was performed to test the difference on HEV as well as 3 ratios between control and case.

	Control	**Case**
Mean/s.e.	(n = 30)	(n = 35)
Total number of HEVs (A)	40.085 / 1.83	41.305 / 1.59
Ratio of dilated HEVs to the total number of HEVs(B/A)	0.149 / 0.0086	0.166 / 0.0186
Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs (C/A)	0.098 / 0.0078	0.101 / 0.0135
Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs(C/B)*	0.646 / 0.0301 **(p=0.0318)**	0.582 / 0.0275

* *p*-value is less than 0.05

The average **C/B** ('Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs') in control groups is statistically significantly higher than that in case group **(p-value = 0.0318)**

3 B. Stages – differences on average HEV and 3 ratios by case/control groups

A non parametric test, Kruskal-Wallis test, was performed to test the difference on the **A** (total no. of HEV) as well as the 3 ratios among different stages.

1) Controls:

		Stage	
Mean/s.e.	1 and 2 (n = 2)	3 (n = 12)	4 (n = 16)
Total number of HEVs (A)	48.625/6.205	44.518/1.98	35.693/2.603
Ratio of dilated HEVs to the total number of HEVs(B/A)	0.127/0.0095	0.148/0.018	0.153/0.0093
Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs (C/A)	0.090/0.0074	0.102/0.0147	0.097/0.0099
Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs(C/B)	0.706/0.006	0.672/0.042	0.619/0.047

Note: Only 1 patient in stage 1 and 1 patient in stage 2

No significant difference has been detected among stages.

2) Cases:

		Stage	
Mean/s.e.	0 and 1 (n = 18)	2 (n = 13)	3 (n = 4)
Total number of HEVs (A)	41.949/2.379	42.606/2.395	34.175/3.524
Ratio of dilated HEVs to the total number of HEVs(B/A)	0.159/0.0146	0.140/0.0133	0.277/0.1475
Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs (C/A)	0.101/0.0129	0.076/0.0095	0.183/0.0986
Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs(C/B)	0.601/0.0339	0.533/0.0548	0.655/0.0398

Note: Only 1 patient in stage 0

No significant difference has been detected among stages.

3 C. Grades – differences on average HEV and 3 ratios by case/control groups

A non-parametric test, Kruskal-Wallis test, was performed to test the difference on the **A** (total no. of HEV) as well as the 3 ratios among different stages in Control Group; A non-parametric test, Wilcoxon-rank-sum test, was performed to test the difference on the **A** (total no. of HEV) as well as the 3 ratios among different stages in Case Group.

1) Controls:

		Grade	
Mean/s.e.	1 (n = 6)	2 (n = 19)	3 (n = 5)
Total number of HEVs (A)	40.575/4.374	40.339/2.059	38.534/6.606
Ratio of dilated HEVs to the total number of HEVs(B/A)	0.1482/0.0265	0.1475/0.011	0.1575/0.0101
Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs (C/A)	0.0961/0.0181	0.0962/0.0109	0.1088/0.0082
Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs(C/B)	0.6339/0.058	0.6353/0.0421	0.6995/0.0571

No significant difference has been detected among grades.

2) Cases:

	Grade	
Mean/s.e.	1 (n = 14)	2 and 3 (n = 21)
Total number of HEVs (A)	42.54/2.652	40.48/2.007
Ratio of dilated HEVs to the total number of HEVs(B/A)	0.1598/0.0181	0.1694/0.0289
Ratio of dilated HEVs with rbcs within its lumen with respect to total no. HEVs (C/A)	0.0985/0.0152	0.1023/0.0204
Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs(C/B)	0.5667/0.0487	0.5921/0.0331

Note: Only 1 patient in grade 3

No significant difference has been detected among grades.

12. Conclusion

In conclusion, our study demonstrates morphologic and functional alterations of the HEVs to become the main blood flow carrier in the lymph node. The analysis reveals the relationship of high endothelial venules and their metamorphosis in pre-metastatic and metastatic environment in regional lymph nodes of tongue cancer patients in correlation with clinical outcomes. Our findings coupled with studies elucidating the basis of lymphangiogesis and angiogenesis within sentinel lymph nodes support our hypothesis that HEV play a pivotal role and may be the elusive junction providing the lymph flow shortcut route into the circulation in sentinel lymph nodes. The confirmation of this shortcut and the exploration of the related molecular mechanism of establishing the shortcut will broaden our knowledge about lymph circulation in cancerous conditions and may provide novel therapeutic targets

13. Discussion

Squamous cell carcinoma of the tongue is one of the most prevalent tumors of the head and neck region, epidemiologically it is also one of the cancers with fast rising rate today affecting especially the young. The prognosis is worse compared to other equivalent carcinomas of the head and neck region as it is one of the cancers with the highest propensity for lymph node metastases. Surgery is the mainstay of treatment to achieve cure for this deadly disease and there are two equally important aspects to the surgical therapy. The adequate resection of the tongue lesion with clear microscopic margins and secondly but more crucially for prognosis ,the surgical clearance of its draining lymphatic basin , in the case of tongue carcinoma, these are the cervical lymph nodes, classified according to anatomical levels 1 to 7.(Illustration 1) Historically, radical neck dissection has been the gold standard for treatment of the neck with regards to LN clearance in surgical treatment of tongue cancers but due to its associated high morbidity, poor patient's tolerance and inherent inferior cosmetic results, neck dissection has evolved to less radical procedures for smaller tumors e.g. supraomohyoid neck dissection and more recently under experimental trials, sentinel lymph node biopsy, following the validating successes of melanoma and breast carcinoma. There are several clinical and pathological factors proposed as prognostic in oral squamous cell carcinoma (SCC), such as stage of primary tumour, site, extension, thickness, grade of histological differentiation and perineural invasion. Of all these clinico-pathological factors, the presence of cervical LN metastasis is the most important factor associated with adverse prognosis. Extracapsular spread in LNs has also been recently identified as a prognostic indicator as well. (Table 2) Tumor recurrence in the neck after radical neck dissection has almost invariably a fatal outcome.

In our series, probably due to our limited and small sample size, we detected a marginal statistical significance when we analyzed the overall survival and disease free interval with respect the tumor stage, grade and tumor volume (Table 5). However, we consistently detected a marginal significance when comparing the 2 groups overall survival based on the presence or absence of LN metastasis. (p-value = 0.066). (Figure 4)

It has been proposed and it is currently being validated that SLN biopsy may play the next stage in the evolution in the neck management and treatment of tongue SCC (32) . Exploration and understanding of this concept coupled with new advances and optimistic results in anti-angiogenesis therapy involving VEGF anti-metabolites translates the next logical exploration to be invested in the study of the pathogenesis of lymph node metastasis. It was shown recently that the lumens of the lymphatic sinuses and blood vessels were dilated in SLN before metastasis. This has been demonstrated lately in xenograft models but cancer physicians and surgeons knew of this fact decades ago, it is called reactive lymphadenopathy, analogous to a reactive lymph node during upper respiratory tract infection. We have demonstrated that lymph nodes are transformed by the primary tongue tumor to become a functional blood vessel–enriched organ before and independent of metastasis, with the changes in the morphology of the HEV to become main blood flow carrier in the lymph node. (Figure 6- high power field photo of dilated HEVs with rbcs inside) This process of vascularization in the LN (i.e. the HEV morphological alterations) appears similar and consistent in human tissues and previous animal models (5) . This study presents functional and structural data that the primary tumor is manipulating lymph nodes' microenvironment and biology to improve the acceptance and proliferation of subsequent tumor deposits leading to established metastases.

In previous studies, the degree of lymphatic dilation in the SLN was significantly correlated with the primary tumor weight, this observation suggests that the lymphatic fluid from the primary tumor induced the persistent alteration of the lymph channel in SLN. These results are consistent with a recent finding that, in contrast to angiogenesis, in which blood flow proceeds only after the vessel develops, lymphangiogenesis can be induced by interstitial fluid channeling (65) . As mentioned, in this aspect this is analogous to immunology studies showing the alteration of lymph channels in SLNs can also occur during an inflammatory reaction, which facilitates the migration of inflammatory cells (66) The difference in cancer metastasis biology and inflammatory process starts here. In endotoxin induced experiments and studies, the dilated lymphatic sinuses and vessels in the associated reactive LNs were full of lymphocytes, but in contrast in tumor-reactive lymphadenopathy, lymphatics contained minimum or no cellular material, suggesting different roles of the SLN lymphatic channels in different pathologic processes. It has been well characterized that the HEVs of lymph nodes play an important role in recruiting lymphocytes for the generation of immune responses. By expressing homing receptors on their surface, which blood lymphocytes can recognize as they pass in circulation, HEV provide a unique location where naive lymphocytes can enter the lymph node (67) .

In a previous study (5), we found that the role of HEV was transformed from a lymphocyte recruiter to become the main blood flow carrier in the SLN prior metastasis. We have shown and verified in this study that in the regional cervical LN, not only could the individual HEV morphology change dramatically to carry more blood flow, but the proliferation rate of HEV endothelial cells was also increased before metastasis. This transformation of HEV as a lymphocytic carrier in LNs to a blood-flow carrier can be seen in large quantity of red blood cells visible in the HEV even in both pre-metastatic regional LNS and more significantly this

phenomenon is also proven to be more pronounced in the regional LNs of the patients with established LN metastasis. We have shown this in our supplementary data that patients with lymph node metastases have more dilated HEVS with rbcs in their LNs as compared to patients without LN metastasis. The average Ratio of dilated HEVs with rbcs within its lumen to total no. of dilated HEVs(C/B) in the control (pN+) group is statistically significantly higher than that in case group (p-value = 0.0318).

In addition, with the benefit of patients' pathological and follow-up information, we have demonstrated statistically that even with an increase of 1 HEV in a high-power-field (HPF), regardless of the group, the risk is 1.024 times worse in terms of overall survival. This is also consistent with clinical knowledge and natural history of the disease. We know for a fact that patients without LN metastases in their regional LNs (i.e. designated as CASES in this study) have a significantly better prognosis; this fact is also reflected in our analysis. (Figure 4 showing the Kaplan Meier curve of OS for cases vs. controls) Cases have a 0.428 times the risk of Controls with regards to overall survival if they have the same number of HEV. It is crucial to realize that this risk is exponential in nature, so to illustrate the significance of this result, we use the following example: Tongue cancer patient A has proven LN metastases in his cervical LN, on further immunohistochemical staining, has zero HEV in a random high-power-field; assuming that everything else (i.e. tumor size, grade, stage, presence of LN metastases etc.) being equal, patient B has 100 HEV in a HPF, pt B will have a [$(1.024)^{100}$ = **10.715**] more than 10 times worse prognosis in terms of overall survival as compared to patient A. Tongue cancer patient C has no LN metastasis and if the HEV amount in a HPF is the same as either patient A or B, patient C's risk as compared to either patient A or B will be **2.34** times (1/0.428= **2.34**) better in

terms of overall survival. Although this did not reach statistical significance (p=0.471), we believe this to be a factor of a small sample size as we continue to demonstrate why this appears to be the case.

Assuming that our hypothesis is true, HEV transformation from a normal immunological mediator to a tumor metastasis mediator is reflected by morphological changes from a normal appearing HEV to a dilated HEV to lastly a dilated HEV containing rbcs. We believe that this metamorphosis is a spectrum and this process begins with the HEV increasing in absolute numbers, then each of them becoming more dilated and lastly every one of them will become a function vessel carrying blood. (Figure 7, 9) This is the reason why we analyzed our data looking at the 3 different stages of HEV transformation (see Material and Methods Section) and the ratio comparing each parameter in an attempt to demonstrate this spectrum. (Illustration 3. Venn diagram illustrating the relationship between the different HEV parameters (**A, B, C**).

In our study, we found that this spectrum of metamorphosis is consistent and verified with the patients' overall survival regardless of their LN metastasis status. We recall that that in the last example we illustrated that a patient 's OS risk is 1.024 times worse than an equivalent patient if there is 1 more HEV in a HPF of his regional LN, the risk is increased to 1.071 if the HEV is a dilated HEV. The risk is even higher at 1.116 times if the HEV is a dilated HEV containing rbcs. This trend is also reflected if we considered all the patients as one cohort (i.e. regardless of LN metastasis status), a patient's OS risk is worsen by 1.004 times if there increase of 1 dilated HEV a in HPF, if that dilated HEV contains rbcs this OS risk in increased to 1.008 times (see section Results 4A.b) .

In the treatment of tongue carcinomas or in any head and neck cancers, disease free interval is as important as it signifies the failure of loco-regional control of which there is little effective therapy. Further surgery e.g. neck dissection to remove loco-regional recurrences is plagued with high morbidity and mortality and this coupled with poor chemotherapy and radiotherapy response rates in recurrences, this more often than not equates to an unfortunate rapid demise of the patient's condition. Disease free Interval (DFI) is also analyzed in the same manner to the three different HEV morphological phenotypes. Normal HEV, Dilated HEV, Dilated HEV with rbcs within its lumens (A, B, C respectively as defined in the Section Patients, Material and Methods).

We found statistical significance in the relationship between the total number of HEV (A) and DFI when we controlled for the group (i.e. taking in the presence of LN metastasis as a factor). ($p=0.022$). This significance is preserved when we analyzed all the patients as a cohort ($p=0.023$). In another words, this translates to mean that the quantity of HEV is inversely related to DFI whether you consider the patient's LN status or choose to disregard it as a factor. If you consider the LN status and take it into statistical consideration, if there is one more HEV in a HPF, the DFI is 1.051 times worse than zero HEV. It is again important to note that the relationship of the quantity of HEV with respect to DFI is exponential in nature. For example, a patient with 100 more HEV (A) in a HPF has $\{(1.051)^{100} = 144.63\}$ 144 times shorter DFI as compared to a equivalent patient with one HEV. Excluding the group effect and consider all the patients as a cohort, in a patient with 1 more HEV in a HPF, the DFI is also about 1.051 times worse than a patient with zero HEV in a HPF ($p=0.023$).

We then analyzed out results further. We looked at the different ratios of abnormal HEV and compared them to several clinic-pathologic parameters namely overall survival, disease–free interval, tumor volume, stage and grade of the tumor. The details results are specified in the results supplementary section.

There is a general trend observed. The more advanced the disease is, the higher the ratio/percentage of abnormality of HEV. This can be seen in when we analyzed OS and the 2 ratios. The OS relative risk worsens by 1.078 if the ratio B/A (ratio of dilated HEVs to the total number of HEV) increased by 1, the OS relative risk worsens by 3.624 times if we consider the ratio of dilated HEVs with rbcs to the total number of HEVs increased by 1. Most importantly, if we consider the most abnormal form of HEV (dilated HEV with rbcs within its lumen, C) and looked at it as a ratio to the total no. of HEV, a patient's OS relative risk worsens by 17.884 times if this ratio (C/A) increase by a factor of 1. This observation approaches marginal significance. (p-value = 0.171). (Figure 8)

In this study, we have shown the relationship of HEV and their transformation in a cancerous environment. We also note that in previous studies the HEV morphology did not alter at all in endotoxin-induced lymphadenopathy, implying a selective reaction of HEV in the cancerous condition. Distinct, differentiated gene expression has also been reported when the endothelial cells respond to the changing of their microenvironment (68) . In a previous study, it was shown that the cellular morphology of the tall endothelial cells forming HEVs changed dramatically to

become flat endothelial cells in the presence of cancer. As a consequence, the HEV was remodeled from a thick-walled, endothelial vessel with a small lumen to a thin walled, large-lumen vessel (Figure 11 and 14), shifting its function from recruitment of lymphocytes to becoming a blood vessel. These facts indicate that the blood vessel endothelium has tremendous potential to adapt its environment. The confined lymph and blood channel alterations within the SLN but not in the next station lymph node imply that an inducer from the primary tumor is functioning locally with the existence of an active primary cancer. VEGF-A has been found to be an inducer of lymphangiogenesis in SLNs (49) . Other studies shown that serum level of VEGF-A was elevated in patients with late-stage NPC (69). VEGF-A is a secreted protein factor that can travel in blood to other lymph nodes, these findings suggest that there may be other inducers involved. It is also of interest to explore the role of HEVs after the establishment of a metastatic tumor nest. The enlarged, remodeled HEVs could integrate into the metastatic tumor vasculature with further differentiation, characterized by the gradual loss of their specific marker MECA-79 from the tumor margin to the central part of the metastatic tumor nest. It has been explained that, compared with primary tumors, the more rapid growth of metastatic lesions in the cervical lymph nodes of NPC patients was due to clonal selection of the cancer cells during metastases, with highly proliferative clones disseminated to the cervical nodes. However, based on our findings, the metastatic tumor vasculature in lymph nodes consists of many large blood vessels derived from normal HEVs, suggesting that the efficiency of nutrition and oxygen supplies could be better for the metastatic tumor cells in the involved lymph node. The enrichment of the blood supply in the lymph node before and after metastasis may favor the growth of newly arriving metastatic cancer cells. Consequently as known in clinical situations seen in the long term follow-up of cancer patients, the involved regional lymph nodes may become manifest, whereas

the primary tumors remain clinically occult for years (70) . Moreover, the high density of functioning blood vessels in lymph nodes may subsequently facilitate the metastasis of cancer cell to distant organs. High endothelial venules being further integrated into the established tumor nests in the LN is a good example of vessel co-option, which is an established and important cancer vasculature development (48)**.** It will be important to elucidate if the highly vascularized premetastatic SLN is associated with an increased metastatic potential. Control of lymphatic fluid movement may also be a target to consider in preventing metastases.

14. Future Directions

If this hypothesis is true about HEV role in cancer metastasis, we can block this remodeling process and see if it prevents distant metastasis. This will confirm the HEV's central role in the pathogenesis of metastasis.

Further studies need to concentrate on the pathways of HEV transformation. A master control gene for the growth and differentiation of HEVs remains to be identified. With regards to this, a recently identified nuclear factor, NF-HEV has potential and is interesting, because it is preferentially expressed by HEVs (71) . Identification and characterization the molecular pathways and the control genes would have considerable clinical applications. It would enable the design of study protocols to induce the formation of HEVs in various tissues, including tumours, improving vaccination strategies against pathogens and cancers. Therefore, there is a wealth of translational applications with regards to targeting HEVs as a ligand for cancer investigational therapeutics. It paves the journey in the discovery of many unknown genes and molecules with potential clinical importance.

15. Limitations

Our study did find some preliminary associations in HEVs with regards disease pathology and clinical correlations; however, it is premature unequivocal conclusions. Our study has its limitations and there are mainly associated with 2 major factors. It is a retrospective study with a limited sample size and its lacks a molecular experimental component.

Since it is an exploratory observational study considering that HEVs role in cancer and metastasis is still in its preliminary phases of full comprehension. The limited sample size of 65 patients (Controls n=28; Cases n= 37) is derived from a 5 year long experience and it is a subset of a large group selected from the Head and Neck database in a large tertiary institution with the oldest and largest surgical department in country (Department of General Surgery, Singapore General Hospital). This sample population size was deemed to be sufficient to show a significant difference in their overall survival between 2 the study groups. Nonetheless, due to the efforts to reduce confounders and to minimize errors and difficulty in the interpretation of our results, a strict selection criteria was applied to have a homogenous group. We thus included only tongue cancer patients are that treated surgically with a component of the corresponding lymphatic system and drainage basin resected and its status proven pathologically with a good documented follow-up period of at least 2 years. Due to the limitations of a small sample size, we believe that some of the associations in our hypothesis did not reach statistical significance due to this fact. The retrospective nature of the study is no doubt a limitation but as the nature of this study does not involve therapeutics or a comparison of 2 factors, the retrospective nature actually works to our advantage. We are able to collate a large sample study size with confirmatory

clinic-pathological data within a short period of time and concentrate our time and effort in the investigative and analytical aspects.

Secondly, one main investigative feature of our study concentrated largely on the histopathological assessment of the patients' lymph node status preserved tissue paraffin blocks in storage. There are limitations to using tissue paraffin sections and the techniques available are limited to date. There are deficiencies in the consistency of the quality of the tissue paraffin blocks, some tissue blocks are less well preserved than others, especially the older blocks may not be stored in optimal conditions over time. We use a self-experimented optimized technique for our immunohistochemistry using anti-MECA-79(also named peripheral node addressin, PNAd) antibody) as our sole antibody for HEV. Ideally we would like to use at least 2 different HEV markers to confirm our findings in event of specificity and sensitivity inaccuracies in the antibody to HEV, however, there is no other commercially available antibody for HEV to date and we do not have the facility and resources to generate a new and more specific antibody for HEV. One assumption in the study is that MECA-79's specificity and sensitivity is high enough and representative of all HEVs in our tissues. Ultimately, we need analysis at the level of molecular genetics will prove to be the critical factor in confirming the value of immunohistochemical stains in the assessment of biological behavior and prognosis. A considerable problem to be overcome is the marked variation in tissue staining that can be encountered, both in different, patients, neoplasms and in different laboratories. These differences reflect the varied biology of neoplasms, as well as differences in fixation and technique. These variations make comprehensive interpretation of the data and results a worthwhile challenge.

A major limitation in the study is the lack of experimental evidence and results to strengthen our observatory data. The next step in confirming our conclusion is to have molecular pathway experiments and studies to elucidate the pathways and the molecular mechanisms underlying the phases of HEVs' metamorphosis peri-metastasis. In order to systematically study the role of the modified HEVs as a blood vessel and a shortcut mechanism for metastasis, our IHC and clinic-pathological correlations are not adequate, we require appropriate tracer molecules with real time imaging technology and xenograft experiments studying the mechanism of the lymph node microenvironment changes correlating to the spreading of cancer cells. As the objective of this study is to establish the presence of a correlation between HEVs and clinico-pathological features in cancer patients, the molecular experiments are not included but are planned for as stated in our future directions.

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