EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND ITS RECEPTOR (VEGFR) IN THYROID NODULAR HYPERPLASIA AND PAPILLARY THYROID CARCINOMA (PTC)

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^{131}I	Radioactive iodine
AJCC	American Joint Committee on Cancer
ASR	Age-Standardised Rate
CR	Crude Rate
CR74	Cumulative Rate
CumR	Cumulative Risk
DAB	3,3′-diaminobenzidine tetrahydrochloride
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EPC	Endothelial precursor cells
Ex	Extrathyroid extension of primary tumours
FGF	Fibroblast growth factor
H&E	Hematoxylin& Eosin
HRP	Horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia
IHC	Immunohistochemistry
LIS	Laboratory Information System
LN-Ex	Extranodal carcinoma extension
M	Distant metastasis
MNG	Multinodular goitre
mRNA	Messenger RNA
N	Lymph node metastasis
ORR	Objective response rate
PIGF	Placental growth factor

PTC	Papillary thyroid carcinoma
RECIST	Response Evaluation Criteria in Solid Tumours
SPSS	Statistical Package for in Social Sciences
T	Primary tumour
TBS	Tris Buffered Saline
Tg	Thyroglobulin
UICC	Union for International Cancer Control
US	United States
USM	UniversitiSains Malaysia
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPF	Vascular permeability factor
WHO	World health organization

ABSTRAK

Latar belakang dan objektif: Vascular Endothelial Growth Factor (VEGF) adalah faktor angiogenik yang memainkan peranan penting dalam pertumbuhan kanser seiring dengan penyelidikan yang berterusan mengenai peranannya sebagai faktor prognosis dan juga disasarkan sebagai agen terapeutik anti-angiogenik dalam kanser tiroid. VEGF diketahui memiliki hubung kaitdengan reseptor VEGF (VEGFR) seperti VEGFR-1 (Flt-1) dan VEGFR-2 (KDR) dalam keadaan patologi seperti yang telah ditunjukkan dalam kajian sebelumnya. Kanser tiroid papilari (PTC) adalah kanser tiroid yang paling banyak ditemui dan kajian menunjukkan insiden kanser yang semakin meningkat dalam bengkak kelenjar tiroid (MNG). Tujuan kajian ini adalah untuk menentukan ekspresi VEGF dan VEGFR dalam bengkak kelenjar tiroid (MNG) dan kanser tiroid papilari (PTC).

Bahan dan kaedah: Satu kajian keratan rentas ke atas 113 kes MNG dan 67 kes PTC diambil dari arkib paraffin blok tisu dari tahun 2003 hingga ke 2014. Tisu sampel telah diposes dan dijalankan ujian immunohistokimia bagi menentukan ekspresi VEGF, VEGFR -1 dan VEGFR-2. Maklumat tentang kanser dalam kelenjar limfa and tisu di luar kelenjar tiroid juga diperolehi.

Keputusan: Purata umur pesakit PTC adalah 44.7 ± 15.8 tahun dan MNG adalah 42.2 ± 13.6 tahun. Pesakit perempuan adalah majoriti bagi kedua-dua diagnosis dengan 86% (97/113) dalam MNG dan 78% (52/67) dalam PTC. Terdapat perbezaan statistik untuk ekspresi VEGFR-1 ($p = 0.028$) dan VEGFR-

2 (p = 0.003) antara MNG dan PTC. Walau bagaimanapun, tiada perbezaan yang signifikan untuk ekspresi VEGF (p = 0.576) antara MNG dan PTC. Ekspresi bersama VEGF dan VEGFR-1 adalah penting dalam kedua-dua MNG (p = 0.016) dan PTC (p = 0.03), sementara itu tidak ada hubungan yang signifikan untuk ekspresi bersama VEGF dan VEGFR-2 (p> 0.05). Pesakit dibahagikan kepada kumpulan muda (<45 tahun) dan kumpulan umur yang lebih tua (≥45 tahun). Kebanyakan pesakit yang positif untuk metastasis kelenjar limfa dan kanser dalam tisu luar tiroid adalah wanita dan daripada kumpulan umur yang lebih tua. Dalam 15 kes PTC yang menunjukkan kanser dalam kelenjar limfa, 60% (9/15) adalah perempuan dan 53% (8/15) berumur 45 tahun dan ke atas. Semua dua kes yang menunjukkan kanser dalam tisu luar tiroid terdiri daripada kumpulan umur yang lebih tua dan perempuan. Tidak ada hubungan yang signifikan (p> 0.05) antara status kelenjar limfa dan kanser dalam tisu luar tiroid dengan kumpulan umur, jantina, VEGF dan VEGFR.

Kesimpulan: VEGF, VEGFR-1 dan VEGFR-2 menunjukkan ekspresi dalam kedua-dua MNG dan PTC. Ekspressi VEGFR-1 dan VEGFR-2 lebih penting dalam PTC dengan keputusan menunjukkan signifikan bagi ekspresi bersama VEGF dan VEGFR-1.

ABSTRACT

Background and objective: Vascular endothelial growth factor (VEGF) is an angiogenic factor that plays important role in tumour growth with ongoing research regarding its role as a prognostic factor and also targeted anti-angiogenic therapeutic agent in thyroid cancer. VEGF is known to have high affinity to VEGF receptors such as VEGFR-1 (Flt-1) and VEGFR-2 (KDR) and their co-expression in pathological condition had been demonstrated in previous studies. Papillary thyroid carcinoma (PTC) is the most common differentiated thyroid cancer and studies showed the increasing incidence of carcinoma arising in multinodular goitre (MNG). The aim of the current study to determine the expression of VEGF and VEGF receptors (VEGFR) in thyroid nodular hyperplasia and PTC.

Materials and methods: We conducted a cross sectional study based on retrieved paraffinized archival tissue blocks of 113 nodular hyperplasia and 67 PTC from the thyroidectomy specimens from the year of 2003 to 2014.The tissue sections were then stained by immunohistochemistry for VEGF, VEGFR-1 and VEGFR-2. The lymph node involvement and extrathyroid extension also were determined.

Results: The mean age of PTC patients was 44.7 ± 15.8years and nodular hyperplasia subjectswere42.2 ± 13.6years. Female gender predominate in both benign nodular hyperplasia and PTC with, 86% (97 out of 113) and 78% (52 out of67) respectively. There was a statistical difference of VEGFR-1 (p=0.028) and

VEGFR-2 (p=0.003) expression between nodular hyperplasia and PTC. However, no significant difference of VEGF expression (p=0.576) between both diseases. Co-expression of VEGF and VEGFR-1 was significant in both nodular hyperplasia (p=0.016) and papillary thyroid carcinoma (p=0.03), meanwhile no relevant relationship for VEGF and VEGFR-2 expression (p>0.05). The patients were further divided into young age group (<45 years old) and older age group (≥45 years old). Most of the subjects positive for lymph node metastasis and had extrathyroid extension were female and from older age group. From 15 lymph nodes with metastasis, 60% (9/15) were female and 53% (8/15) from older age group. All two cases that showed extrathyroid extension were from older age group and female. However, there was no significant association (p>0.05) between lymph node status and extrathyroid extension with age groups, gender, VEGF and VEGFR expression.

Conclusions: VEGF, VEGFR-1 and VEGFR-2 showed overexpression in both benign thyroid nodular hyperplasia and PTC. The expression of VEGFR-1 and VEGFR-2 more significant in PTC with relevant co-expression of VEGF and VEGFR-1.

CHAPTER 1

INTRODUCTION

1.1 Thyroid carcinoma

Thyroid carcinoma is a relatively rare tumour, but it represents the most frequent form of cancer of the endocrine glands. It represents about 1% of all human malignancies and about 90% of all endocrine tumours. Papillary thyroid carcinoma (PTC) represents about 90% and follicular thyroid carcinoma the 10% of differentiated thyroid carcinoma. The annual incidence of thyroid cancer has been reported to range between 1.2 and 2.6 cases per 100,000 in men and 2.0-3.8 cases per 100,000 in women **(Taccaliti *et al.*, 2012)**. An estimated 60,220 new cases of thyroid cancer are expected to be diagnosed in 2013 in the United States (US), with 3 in 4 cases occurring in women. The incidence rate of thyroid cancer has been increasing sharply since the mid-1990s, and it is the fastest increasing cancer in both men and women. From 2005 to 2009, incidence rates increased by 5.6% per year in men and 7.0% per year in women.

1.1.2 Classification.

Table 1.1: Histological classification of thyroid tumours.
Source : World Health Organization (WHO) Pathology and Genetics of Tumours of Endocrine Organs (DeLellis and Williams, 2004).

Papillary carcinoma

Follicular carcinoma

Poorly differentiated carcinoma

Undifferentiated (anaplastic) carcinoma

Squamous cell carcinoma

Medullary carcinoma

Sclerosing mucoepidermoid carcinoma with eosinophils

Mucinous carcinoma

Mucoepidermoid carcinoma

Mixed medullary and follicular cell carcinoma

Spindle cell tumour with thymus-like differentiation

Carcinoma showing thymus-like differentiation

The interest of this study is papillary thyroid carcinoma. Papillary thyroid carcinoma is the most common type of malignant thyroid tumour and there has been an increasing incidence of papillary thyroid carcinoma worldwide for the past few decades **(Witmer *et al.*, 2002).**

1.1.3 Etiology of thyroid carcinoma

Epidemiologically ascertained risk factors of thyroid cancer are ionising radiation, the presence of thyroid adenoma and multinodular goitre. Multinodular goitre (MNG) is defined as the palpation of multiple discrete nodules in the enlarged thyroid gland **(Shrestha and Shrestha, 2014)**. Multinodularity of goitre should no longer be considered an indicator of probable benign disease **(Anwar *et al.*, 2011)**. In published reports, the incidence of carcinoma in multinodular goitre is reported with a percentage that varies from 10% **(Hanumanthappa *et al.*, 2012)**, 13 % **(Shrestha D and Shrestha S 2014),** 13.7 % **(Gandolfi *et al.*, 2004)** to 14.4% **(Anwar *et al.*, 2011).**

In most studies, among the malignancies in multinodular goitre, papillary carcinoma was the most common followed by follicular carcinoma and anaplastic carcinoma**(Anwar *et al.*, 2011; Shrestha and Shrestha, 2014)**. These studies show the prevalence of cancer is significant in nodular goitres and these malignant tumours are usually of the papillary type. Endemic goitre is a major health concern in many parts of the world including Malaysia. Seven states in the country have been noted to have high incidence of goitre, including Kelantan **(Ministry of Health, Malaysia, 1998).**

Apart from radiation exposure as an aetiological factor in the thyroid cancer, major advances have been made in the past decade in the understanding of the molecular mechanisms involved in the initiation and progression of thyroid

carcinoma. Genes thought to be involved in papillary and follicular carcinomas include the gsp, ret, trk, ras, met and p53 oncogenes. Activation of the ret protooncogene located on chromosome 10 is critical in the initiation of papillary and medullary carcinoma while the p53 and N-ras may be important for progression of well differentiated thyroid carcinomas **(Yu *et al.*, 2005).**

1.1.4 Treatment and prognosis

Treatment of differentiated thyroid cancer ususally involves surgical resection of the tumour either total or near total thyroidectomy. In many cases, it is followed by radiotherapy or radioactive Iodine (^{131}I) therapy **(Lazarus and Obuobie, 2000; Mazzaferri, 2006).** Thyroid remnant ablation is defined as ^{131}I therapy administered to destroy presumably normal residual thyroid tissue **(Mazzaferri, 2006).** The measurement of serum thyroglobulin (Tg) is the most sensitive marker of persistent disease and is used routinely as tumour marker in the follow up of these patients. However, the serum Tg concentration is unreliable when a large thyroid remnant is present **(Lazarus and Obuobie, 2000; Mazzaferri, 2006)**. Apart from thyroidectomy, modified neck dissection is usually advisable in the present of cervical lymph node metastases **(Mazzaferri, 2006).**

Tumour staging of thyroid carcinoma relies on age of patients, size of primary tumour, extrathyroidal spread, and regional and distant metastases. These criteria play an important role as prognostic factors. The application of TNM

system in staging of thyroid carcinoma is endorsed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). In the TNM system, each cancer is assigned a letter or number to describe the primary tumour (T), lymph node involvement (N), and metastases (M) **WHO Pathology and Genetics of Tumours of Endocrine Organs (DeLellis and Williams, 2004).**

1.2 Angiogenesis

Angiogenesis is the process of new blood vessels formation from pre-existing microvasculature, primarily venules. The process of growth and expansion of the vasculature occurs in response to injury, ischaemia and also involved in pathological conditions like tumour growth. Angiogenesis promote the tumour to increase in size beyond the constraints of the original blood supply. This is an important process in the growth and metastasis of many cancers **(E.Johnson and Wilgus, 2012; Klein *et al.*, 2001; Kumar *et al.*, 2013)**. Typically, angiogenesis is required for tumours to grow beyond 1 to 2mm in size and offers a route for tumour cells to disseminate to secondary sites **(Folkman, 2002)**. For tumour development and progression, many researchers have been looking into apoptotic and angiogenic activities of cancer cells **(Younes *et al.*, 2006)**. Tumour cells are thought to secrete angiogenic factors to induce neovascularization around tumours **(Katoh *et al.*, 1999)**.

1.2.1 Vascular Endothelial Growth Factor (VEGF).

Vascular endothelial growth factor (VEGF) is a potent pro-angiogenic factor which it has the ability to induce angiogenesis **(Dvorak *et al.*, 1995; E.Johnson and Wilgus, 2012).** VEGF is also known as vascular permeability factor (VPF) and it is one of the vascular permeabilizing agent **(Dvorak *et al.*, 1995; Katoh *et al.*, 1999)**. The accumulation of VEGF on the endothelium of tumour blood vessel has been associated with hyperpermeability of microvessels, primarily postcapillary venules and small veins for macromolecules **(Dvorak *et al.*, 1995; Katoh *et al.*, 1999)**.

1.2.2 VEGF Receptors (VEGFR)

There are 3 types of vascular endothelial growth factor receptors (VEGFR) with high affinity to VEGF which are known as VEGFR-1 (Flt-1), VEGFR-2 (KDR in human and Flk-1 in mice) and VEGFR-3 (Flt-4, lymphatic endothelial cells). VEGFRs are members of a receptor tyrosine kinase family which localized endothelial cells during embryonic development. VEGFRs mediate signaling in endothelial cells of blood and lymph vessels, which induce cell proliferation, survival, or differentiation. This signaling is required for normal development and maintenance of the vascular bed and for angiogenic responses under (patho)physiological conditions **(Witmer *et al.*, 2002)**.

1.2.3 VEGF and targeted therapy

Angiogenesis is of central importance in tumour growth and progression as it involves in sprouting of new vessels from existing blood vessels. As a result, tumour angiogenesis is a target of cancer therapy **(Katoh *et al.*, 1999; Kumar *et al.*, 2013).** The vascular endothelial growth factor (VEGF) family is involved in one of the steps in angiogenesis, which it increase permeability. Therefore VEGF is considered as a potential target for anti-angiogenic therapeutic strategies against cancer **(Witmer *et al.*, 2002).**

CHAPTER 2

LITERATURE REVIEW

2.1 Incidence of malignancy in multinodular goitre.

Multinodular goitre also termed nodular hyperplasia has been cited in the literature as an associated factor of thyroid cancer, particularly papillary carcinoma **(Anwar *et al.*, 2011; Gandolfi *et al.*, 2004; Shrestha and Shrestha, 2014).**

In the management of nodular goitre, the primary challenge is to rule out malignancy **(Anwar *et al.*, 2011).** It may present either as a solitary nodule or as a dominant nodule in a multinodular goitre. Thyroidectomy is the choice of treatment in solitary or multiple nodules which produce pressure symptoms or become cosmetically unacceptable. Those nodules which are suspected of malignancy are similarly best treated by surgery **(Anwar *et al.*, 2011).**

Various studies have reported the incidence of malignancy in multinodular goitre. **Anwar *et al.* (2011)** in 204 cases of nodular goitres found a frequency of malignancy in 16.18% of their cases. The authors further divided the nodular goitres into two categories; (1) solitary thyroid nodule when there was a single nodule palpable in the gland on clinical examination and (2) multinodular goitre

when more than one nodule was detected on examination. The prevalence of malignancy was higher in solitary thyroid nodule (24.32%) compared to multinodular goitre (14.37%). This finding supports that solitary thyroid nodules have high a possibility of being malignant; however multinodular goitres also demonstrate the potential risk of malignancy. The distribution type of tumour also shows different in both solitary thyroid nodule and multinodular goitre. Follicular carcinoma (15.2%) was the commonest malignancy encountered in solitary thyroid nodules and papillary carcinoma (48.5%) was more frequent in multinodular goitres.

In a study reported in 1996, histological examination of 300 consecutive cases of multinodular goitre specimens seen at Hospital Universiti Sains Malaysia (USM), 34% of them had malignant transformation **(Madhavan and Othman, 1996)**. **Hanumanthappa *et al.* (2012)** studied 100 multinodular goitre cases and 10 (10%) cases contained malignant foci. Among them, papillary thyroid carcinoma (60%) was the most common type of malignancy. **D. Shrestha et al. (2014)** found the frequency of malignancy in the multinodular goitre was 13%. Among the malignancies, papillary carcinoma was the most common (84.61%) followed by follicular carcinoma and anaplastic carcinoma **(Shrestha and Shrestha, 2014).**

2.2 Prognostic factors in papillary thyroid carcinoma

There are few prognostic markers that being used among researchers in their reported series of thyroid cancer such as age, tumour size, extrathyroidal extension, distant metastasis, lymph node metastasis, gender and multifocality of thyroid cancer **(Shaha, 2006).**

Ito *et al.* (2011) investigated four prognostic factors of papillary thyroid carcinoma which are important for predicting a patient's prognosis. The factors were tumour size more than 4cm (T), lymph node metastasis measuring 3cm (N), extrathyroid extension of primary tumours (Ex) and extranodal carcinoma extension (LN-Ex). All these factors were evaluated from preoperative assessment and also as the results of intraoperative findings. In this study, they investigated the differences in the significance of these prognostic factors according to patient age and sex. They further dividing the subjects into four groups based on age and sex: (1) older women (age ≥ 55 years); (2) older men (age ≥ 55 years); (3) younger women (age < 55 years); and (4) younger men (age <55 years). They studied the association of patient's groups and prognostic factors with local recurrence, distant recurrence and carcinoma death. They found that the significance of prognostic factors of papillary thyroid carcinoma varied according to patient sex and age. The findings might contribute not only to evaluating the prognosis but also to deciding therapeutic strategies for each patient. The prominent prognostic factor for local and distant recurrences in older patients (≥ 55 years old) was extrathyroid extension of

primary tumours (Ex). This will alarm the surgeon to perform a careful and extensive excision of the site of carcinoma extension and extensive lymph node dissection, especially for older patients with was extrathyroid extension of primary tumours (Ex).

2.3 Expression of VEGF and it's receptors in differentiated thyroid cancer and benign thyroid.

2.3.1 Angiogenesis and role of VEGF and it's receptors.

Blood vessel formation is also known as angiogenesis or neovascularization. Angiogenesis occurs by mobilization of endothelial precursor cells (EPCs) from the bone marrow and from pre-existing vessels (Figure 2.1). In angiogenesis from pre-existing vessels, the extension and branching initiated by motility and proliferation of endothelial cells to form the capillary sprouts. The mechanisms include vasodilation and increased permeability of the existing vessels, degradation of extracellular matrix (ECM), and migration of endothelial cells. One of the major step is vasodilation in response to nitric oxide and VEGF-induced increased permeability of the pre-existing vessel **(Kumar *et al.*, 2010b).**

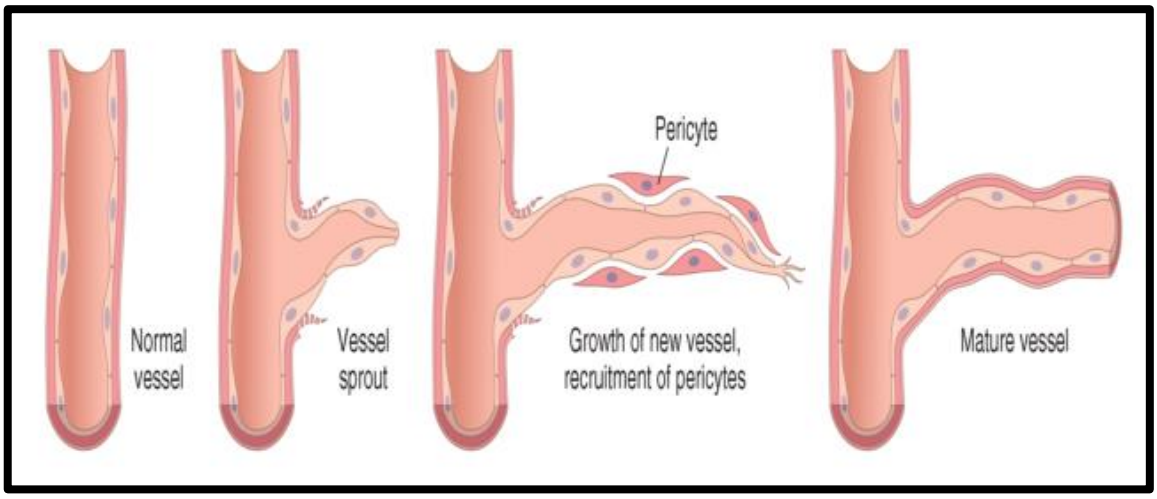


Figure 2.1: Mechanism of angiogenesis from pre-existing vessel occurs mainly by growth factor-driven outgrowth of residual endothelium, sprouting of new vessels, and recruitment of pericytes to form new vessels.
Source: Robbins & Cotrans: Pathologic Basis of Disease. Kumar et. al. 2013, 9th edition.

Angiogenesis plays a pivotal role in the pathogenesis and growth of differentiated thyroid cancer. The relevance of tumour angiogenesis in thyroid tumour is shown by significant correlations between microvessel density and increasing size of primary tumour, intrathyroid tumour spread, and disease-free survival. The importance of angiogenesis in tumour progression and metastasis is well-recognized and its potential role in prognosis and therapeutic application has led to many ongoing clinical researches **(Younes *et al.*, 2006).**

VEGF plays a crucial role as growth factor in adult tissues for vascular development and neovascularization in physiologic angiogenesis and pathological processes occurring in chronic inflammation, wound healing, tumours, and diabetic retinopathy **(Cross *et al.*, 2003; Kumar *et al.*, 2010b).** VEGF promotes angiogenesis, induces migration and proliferation of endothelial

cells, and increases vascular permeability **(Durante *et al.*, 2011; Kumar *et al.*, 2010b; Lennard *et al.*, 2001).**

There are several growth factors contribute to angiogenesis include VEGF, Fibroblast growth factor (FGF) and Angiopoietins. The VEGF family of growth factors includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E and placental growth factor (PlGF) **(Kumar *et al.*, 2013)**. VEGF-A, the most prominent member of the VEGF family, is generally referred to as VEGF and is one of the key regulators of angiogenesis, including after injury and also the promotion of tumour progression and metastasis **(Durante *et al.*, 2011; Kumar *et al.*, 2013).** VEGF-B and PlGF are involved in vessel development in the embryo. Lymphangiogenesis and angiogenesis are stimulated by VEGF-C and VEGF-D. VEGFs are expressed in most adult tissues, with the highest expression in epithelial cells adjacent to fenestrated epithelium such as podocytes in the kidney and pigment epithelium in the retina **(Kumar *et al.*, 2013).**

VEGF-A, the original VEGF, exists in four different isoforms (comprising 121, 165, 189 and 206 amino acids in humans), which are generated by alternative splicing of a single pre-mRNA species. The region encoding VEGF-A spans,14kb and contains eight exons (Figure 2.2). The isoforms differ in their ability to bind to heparan sulphate and extracellular matrix (ECM) **(Cross *et al.*, 2003).**

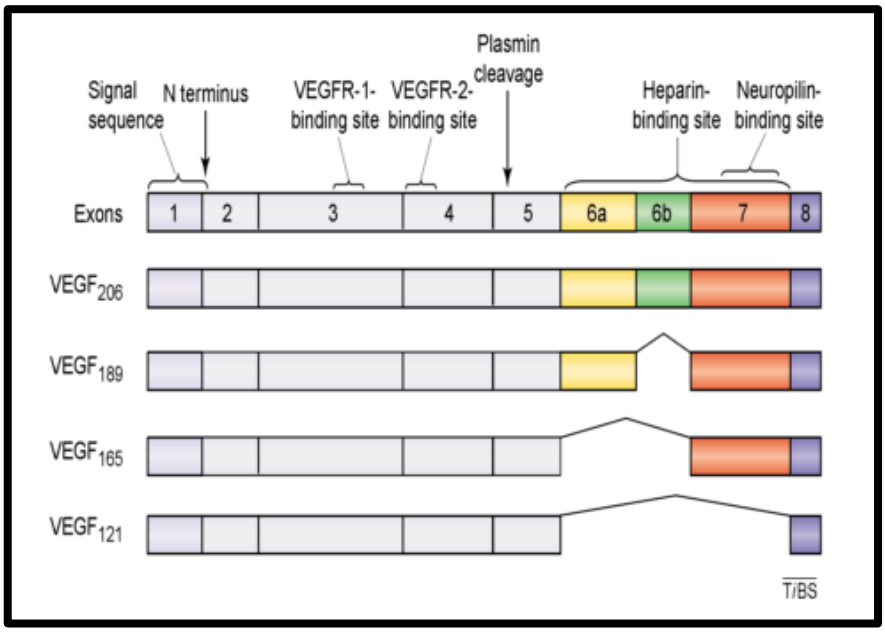


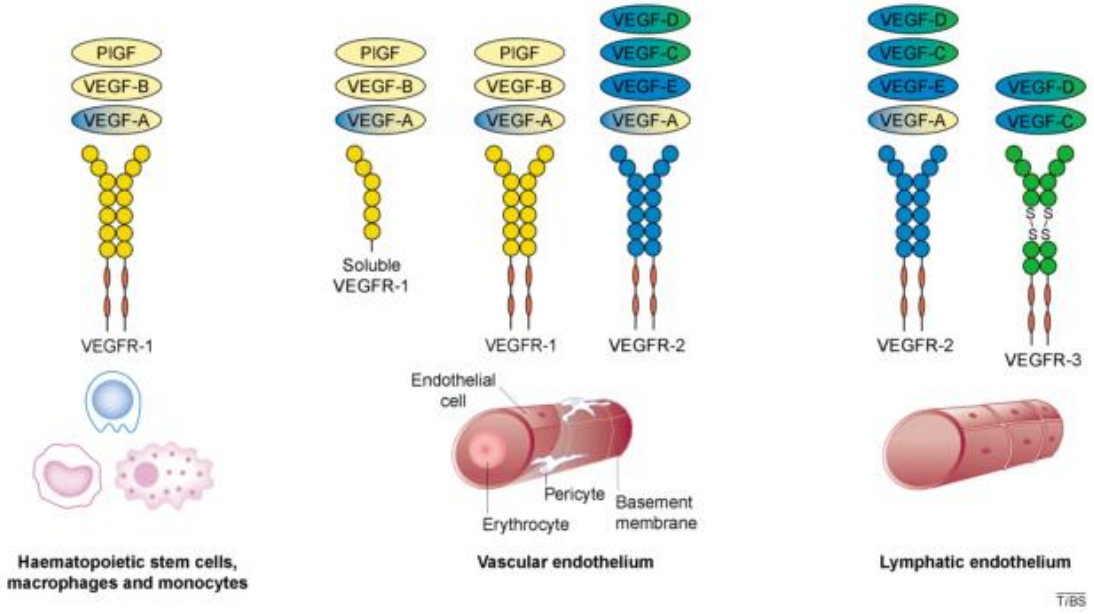
Figure 2.2: Exon structure of the vascular endothelial growth factor VEGF-A mRNA splice variants. The gene encoding VEGF-A consists of eight exons that encode several different structural motifs. Alternative splicing of a single pre-mRNA species produces at least four different VEGF-A isoforms that vary in total amino-acid number. In humans these correspond to VEGF-A121, VEGF-A165, VEGF-A189 and VEGF-A206, of which VEGF-A165 is the predominant form.
Source : Cross *et al.,* 2003

Hypoxia is an important inducer and regulator of VEGF expression **(Cross *et al.*, 2003; Kumar *et al.*, 2013).** VEGF expression is implicated in several diseases that are characterized by excess angiogenesis include cancer. Thus, most of the tumours that express VEGF, leading to vascularization of the tumour and expansion of the tumour mass**(Cross *et al.*, 2003).**

VEGF bind to a family of tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3). VEGF-A is generally binds to VEGFR-1, VEGFR-2 and VEGFR-3. VEGF-B and PlGF bind only to VEGFR-1; meanwhile VEGF-C and VEGF-D are expressed as propeptides that primarily bind to VEGFR-**3 (Cross *et al.*, 2003)**.

In pathological conditions, VEGF activity accommpany the increased levels of VEGF receptors expression including VEGFR-1, VEGFR-2, and VEGFR-3 **(Witmer *et al.*, 2002).** Of the various receptors for VEGF, VEGFR-2 is the most important in angiogenesis. It is expressed by endothelial cells and their precursors, by other cell types, and by many tumour cells **(Cross *et al.*, 2003; Jalali Nadoushan *et al.*, 2012; Kumar *et al.*, 2010b; Witmer *et al.*, 2002).**

There are two high-affinity VPF/VEGF receptors have been described in vascular endothelium that are Flt-1 (VEGFR-1) and KDR (VEGFR-2). Both are transmembrane protein with cytoplasmic tyrosine kinase domains **(Sherma, 2003)**. At the sites of VPF/VEGF overexpression, both Flt-1 and KDR are commonly and strikingly overexpressed in adjacent microvascular endothelial cells. Examples include various human and animal tumours **(Sherma, 2003).**



Schematic illustration of vascular endothelial growth factor receptor (VEGFR) expression patterns and ligand specificity. The VEGF ligand family includes VEGF-A, -B, -C, -D, -E and placenta growth factor (PIGF), which all bind in a specific manner to three different receptor tyrosine kinases, VEGFR-1,-2 and -3. The extracellular domain of VEGFR-1 is also expressed as a soluble protein. VEGFR-1 and its ligands are yellow, VEGFR-2 and its ligands are blue, and VEGFR-3 and its ligands are green. Ligands shaded in two colours bind to more than one receptor type. Both PIGF and VEGF-B are selective ligands for VEGFR-1, whereas VEGF-E is a selective ligand for VEGFR-2. Unprocessed VEGF-C and -D are selective ligands for VEGFR-3. By contrast, all VEGF-A isoforms bind to both VEGFR-1 and VEGFR-2, and processed VEGF-C and VEGF-D ligands interact with both VEGFR-2 and VEGFR-3. VEGFR-1 is expressed in haematopoietic stem cells, macrophages and monocytes as well as on the vascular endothelium. VEGFR-2 is expressed on both the vascular and lymphatic endothelium, whereas VEGFR-3 is predominantly expressed on the lymphatic endothelium. Vascular endothelium shows endothelial cells with tight inter-endothelial junctions surrounded by a continuous layer of basement membrane. The vessel is stabilized by specialized smooth-muscle cells, denoted pericytes. By contrast, the lymphatic endothelium lack inter-endothelial junctions, basement membrane and supporting pericytes, all of which contribute to the permeability of lymphatic vessels to interstitial fluid. Lymphatic endothelial cells are anchored to the surrounding tissue by filaments.

Figure 2.3: Schematic illustration of vascular endothelial growth factor receptors (VEGFR) expression pattern and ligand specificity.
Source: Cross *et al.*, 2003

VEGF is an angiogenic factor and its expression has been demonstrated in thyroid tissue.

Soh *et al.* (1997) conducted a study to look for expression of VEGF messenger RNA (mRNA) and production of VEGF protein in cell lines from human follicular carcinoma, papillary carcinoma, medullary thyroid carcinoma, Hurthle cell cancers, follicular adenoma and Graves' thyroid tissue. They found that normal, hyperplastic, and neoplastic thyroid tissues all were expressing VEGF mRNA, and also secreting VEGF protein. However, the expression of VEGF mRNA was higher and the intensity of VEGF stain was stronger in thyroid cancer cells than normal thyroid cells. In comparison between follicular tumour cell origin and parafollicular tumour cell origin, the results showed higher VEGF mRNA expression and stronger intensity of VEGF stain in the follicular tumour cells. The higher levels of VEGF expression in differentiated thyroid cancers of follicular cell origin suggests a role in oncogenesis.

Vieira *et al.* (2005) conducted a study to look for expression of VEGF and VEGF receptors (VEGFR) in thyroid carcinomas of follicular origin as well as their role in thyroid carcinogenesis. They demonstrated that VEGF, VEGFR-1 and VEGFR-2 were more expressed in papillary thyroid carcinoma compared to follicular thyroid carcinoma and poorly differentiated thyroid carcinoma. The expression of VEGF was significantly ($p<0.05$) more prevalent in papillary thyroid carcinoma (79%) compared to follicular thyroid carcinoma and poorly differentiated thyroid carcinoma. They also found higher expression of VEGFR-1 and VEGFR-2 in papillary thyroid carcinoma with 76% and 68% of cases, respectively. Their study showed the correlation between VEGF expression and microvessel density was statistically significant in papillary thyroid carcinoma, but not in follicular thyroid carcinoma.

2.3.2 Difference of VEGF and it's receptors expression in thyroid nodular hyperplasia and papillary thyroid carcinoma.

Jebreel *et al.* (2007) found that VEGF was co-expressed with its receptors, VEGFR-1 and VEGFR-2, in both benign and malignant thyroid disease. In the study, the specimens are comprising of normal thyroid tissue, multinodular goitre, Grave's disease, Hashimoto's thyroiditis, follicular adenoma and papillary thyroid carcinoma with 92% of all thyroid tissue studied were positive for VEGF staining. There was an increase in both the distribution and intensity of VEGF staining in multinodular goitre, follicular adenomas and papillary carcinoma. They studied 7 cases of papillary thyroid carcinoma and 17 cases of multinodular goitre. For papillary thyroid carcinoma, they showed that 100% were positive for VEGF expression, 86% were positive for VEGFR-1 expression and 100% were positive for VEGFR-2 expression. In multinodular goitre, there were 94% positive for VEGF expression, 94% were positive for VEGFR-1 expression and 100% were positive for VEGFR-2 expression. Their findings also showed that VEGFR-1 and VEGFR-2 were widely expressed in the thyrocytes of both benign and neoplastic thyroid diseases; however the receptors showed minimal variation in co-expression with VEGF in the vascular endothelium. This suggesting that the up-regulation of VEGF and not its receptors occurs as tissue becomes autonomous.

In a study performed by **Katoh *et al.* (1999)**, 25 out of 28 thyroid tumour tissues were positive for VEGF including 9 out of 10 papillary thyroid carcinomas. They reported that the expression of both VEGF mRNA and VEGF protein immunohistochemically were identical in cells covering papillary areas. The

intensity of staining is more than that in the follicle-forming cell. These findings suggest the relation of VEGF and morphogenesis of papillae of papillary thyroid carcinoma. Focal endothelial staining was also noted in normal and neoplastic thyroid tissue.

2.3.3 Expression of VEGF and it's receptors in papillary thyroid carcinoma associated with prognostic factors.

Jiang et al. (2005) studied 115 cases of papillary thyroid carcinoma and 20 cases of nodular goitre. They reported that VEGF expression was significantly higher in papillary thyroid carcinoma (PTC) than in nodular goitre and closely related to the size of papillary thyroid carcinoma. They also found VEGF-C and VEGF-D are closely related to lymph node metastasis of papillary thyroid carcinoma.

Jalali Nadoushan *et al.* (2012) studied 99 cases of papillary thyroid carcinoma (PTC). They showed that 40.2% were positive for VEGFR expression. They concluded that there was no meaningful relationship between VEGFR expression and tumour size and gender of patients, but this relationship was seen between the expression of VEGFR and lymph nodes involvement.

In a study conducted by **Lennard *et al.* (2001)**, they correlate the VEGF expression with the survival in papillary thyroid carcinoma. They studied 96 subjects of papillary thyroid carcinoma with 98% show predominantly of slight-to-moderate intensity of VEGF expression in the majority of the malignant cells. The pattern of VEGF staining in the initial surgical specimen is strongly associated with the incidence of local and distant metastasis in papillary thyroid carcinoma. These findings support that the intensity of VEGF expression is associated with increased risk of recurrence and decreased disease-free survival in papillary thyroid carcinoma.

An increased in VEGF expression has been proposed as the prognostic marker in differentiated thyroid cancers; specifically papillary thyroid carcinoma in the study conducted by **Jalali Nadoushan *et al.* (2012)**. An aggressive disease could have an increased VEGF production which indicates the ability of the tumour to metastasize. The final score of VEGF immunostaining was obtained by the multiplication of intensity score and proportion score (percentage of positivity). From the discriminant analysis, a value of 6 or more indicates that the tumours are at high risk of metastasis. This information can guide the physician for proper and close follow up schedule in the management of the patient.

2.4 Targeted therapy on VEGFR.

Tyrosine kinase inhibitor is one of the interest researches in the targeted therapy in advanced thyroid cancer.

Vieira *et al.* (2005) had concluded that VEGF autocrine action in thyroid carcinoma could play a crucial role in tumour cell survival and could represent a useful therapeutic target for thyroid tumours. They found that the blockade of either VEGF or its receptors with neutralizing antibodies significantly reduced cell viability (live/dead ratio) by 40% to 60%, increased apoptosis and delay in cell-cycle progression (increased in modest arrest of cells in in G_0+G_1 phase and decrease of cells in G_2 phase) of the VEGFR-positive thyroid tumour cell line NPA'87.

Axitinib is an oral, potent, and selective inhibitor of vascular endothelial growth factor receptors (VEGFR) 1, 2, and 3 in patients with advanced thyroid cancer. Axitinib was selected as the interest drug in the study by **Cohen *et al.* (2008).** Their objective was to assess the activity and safety of axitinib. They conducted a study on patients with advanced thyroid cancer of any histology with the most common type was papillary carcinoma, accounting for 30 (50%) of 60 cancers. The inclusive criteria was patients that resistant or not appropriate for ^{131}I (iodine) treatment.

The primary assessment in their study was objective response rate (ORR) by Response Evaluation Criteria in Solid Tumours (RECIST). They demonstrated the results with the high response rate, prolonged duration of response, and overall survival. Most patients experienced some tumour shrinkage during axitinib treatment, with 5 out of 9 patients who treated with prior chemotherapy showed partial response and maximum tumour regression range of 36% to 54%. Moreover, 38% of patients experienced stable disease for 16 weeks or more by RECIST, and the median progression-free survival time in excess of 18 months. These results are notable given that majority of patients were men and had metastatic disease, which are risk factors for poor prognosis.

Furthermore, they also demonstrated that axitinib is an oral inhibitor targeting VEGFRs. They investigated the effect of the drug on soluble proteins as exploratory pharmacodynamics markers. Axitinib was selectively decreased sVEGFR-2 and sVEGFR-3 plasma concentrations versus sKIT (stem-cell factor receptor). **Cohen *et al.* (2008)** concluded that axitinib has generally favourable safety profile and is a selective inhibitor of VEGFR with antitumour activity in all histologic subtypes of advanced thyroid cancer and also in patient with limited therapeutic options.

(Younes *et al.* (2006)) reported that simultaneous blockade of Epidermal growth factor receptor (EGFR) and Vascular endothelial growth factor receptor (VEGFR) signalling by AEE788 alone or in combination with paclitaxel can

significantly reduce follicular thyroid carcinoma tumour volume in nude mice by both direct antitumor and antiangiogenic effects.

CHAPTER 3

AIMS AND OBJECTIVES

3.1 Rational of the study

Papillary thyroid carcinoma is the most common type of thyroid malignancy, which represents 90% of differentiated thyroid carcinoma with excellent overall survival, with a 10-year survival rate in excess of 95% **(Kumar *et al.*, 2010a; Taccaliti *et al.*, 2012).** VEGF is participating in the induction and progression of neoplastic process involving the thyroid gland, by playing a key role in vascularization of solid tumours **(Konturek and Barczynski, 2012).** Increased VEGF expression has been associated with poor clinical outcomes in many malignancies. Several recent reports documented over expression of VEGF in papillary thyroid carcinoma **(Lennard *et al.*, 2001)**. Overexpression of VEGF and it's receptors could guide the physician in close and proper follow up of the patient. By understanding the underlying process of angiogenesis, it has been a promising therapeutic target to inhibit tumour angiogenesis **(E.Johnson and Wilgus, 2012; Kumar *et al.*, 2013).**