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Anaplastic thyroid cancer Irish epidemiology and novel chemotherapeutic strategies

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**‘Anaplastic Thyroid Cancer
Irish Epidemiology
and
Novel Chemotherapeutic Strategies’**

Awarded Thesis

By

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To the National University of Ireland

For the Post Graduate Research Degree of Doctor of Medicine



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Work carried out in
The Department of Otolaryngology, Head and Neck Surgery
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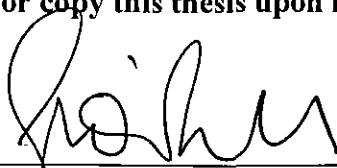
Declaration

I declare that this present work has not previously been submitted as an exercise for a degree at this or any other University.

This thesis consists entirely of my own work except where references indicate otherwise.

The library's of The University College Dublin or Royal College of Surgeons in Ireland may lend or copy this thesis upon request.

Signed:



Date:

Nov 4th 2009

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Abbreviation Table

17AAG:	17 allylamino-17-demethoxygeldanamycin
AJCC:	American joint committee on cancer
ATC:	Anaplastic thyroid cancer
DAHNO:	Data for head and neck oncology database
DMSO:	Dimethyl sulfoxide
EGFR:	Epidermal growth factor receptor
GA:	Geldanamycin
IARC:	International agency for research in cancer
ER-1:	Oestrogen receptor
Her-2:	Epidermal growth factor receptor II
Hsp:	Heat Shock Protein
IHNOD:	Irish head and neck oncology database
MRP:	Multidrug resistance
MTT:	Dimethyl thiazol-diphenyltetrazolium bromide
NCR:	National cancer registry
NSAID:	Non steroidal anti inflammatory drugs
P-gp:	P-glycoprotein
Rb:	Rhetinoblastoma Protein
SERMS:	Selective estrogen receptor modulator
VEGF:	Vascular endothelial growth factor

Academic awards related to this body of work

‘Sir Angell – James’ Prize

Otolaryngology Research Society (ORS)

Plenary Prize

London, September 2005

‘The Sheppard Prize’

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Executive Summary

This body of work was conducted over a four year period. Within this timeframe we have conducted a National Epidemiology project, established a National Head and Neck Cancer database and completed Oncology laboratory investigations.

Anaplastic thyroid cancer (ATC) is the most aggressive endocrine disease in nature. Within the thyroid gland a heterogeneous group of neoplasms may develop. These can range from well differentiated tumours with an excellent prognosis, to ATC tumours which present with distant dissemination of disease in 20-50% of cases, adjacent tissue invasion in 90% of cases, have a reported tumour volume doubling time of one week, and place the patient at a very real risk of death attributed to upper airway obstruction and suffocation. The mean survival is approximately 3-7 months however the most important prognostic factor appears to be disease burden at the time of diagnosis. The high percentage of dissemination highlights the need for effective systemic chemotherapy and prompted a 'Lancet' editorial in 2005 to quote 'There is a pressing need for novel chemotherapeutic strategies in the treatment of anaplastic thyroid cancer'.

Thyroid cancer has undergone a seismic epidemiology shift in the last 30 years. The incidence of thyroid cancer has risen 2.4 fold over this period. In an Irish context we began our project by estimating the impact ATC has had on this country. Following acquisition of consent from twenty eight Irish consultants (whom the patients are tracked to, according to The National Cancer Registry, NCR) we analyzed the registry data, and

patients' charts to examine the epidemiology of ATC in Ireland between 1994 and 2004. Clinically relevant data regarding gender distribution, age, diagnosis, treatment and survival was considered.

A total of 51 cases were confirmed in this period representing 6.3% of all Thyroid cancers for this period. Males present earlier with mean age 66 years old, females at 72 years old. Analysis revealed a gender predominance of females (64.7%). Average survival was 3.8 months. 49% of patients never smoked however, the remainder who did presented a decade earlier. We report no statistical impact of any treatment option upon survival.

Further work is necessary on the NCR data uptake to improve the quality of clinically relevant information amenable for audit analysis. We then established the IHNOD project, Irish Head and Neck Oncology Database to receive oncology details from all head and neck cases presenting in the Republic of Ireland. This Database marks a commitment to improved audit evaluation of our patients which we believe will impact on administrative, medical and surgical aspects of care. The National database has been launched since January 08.

Our epidemiology results confirmed ATC is a fatal malignancy and suggest a higher prevalence of this disease in Ireland than international statistics. The gender imbalance may direct clinical and laboratory research towards an endocrine treatment approach.

Armed with this information we started our laboratory investigations by performing immunohistochemical analysis of the Estrogen Receptor -1, EGF-R1 and Her2/neu expression in ATC. This was examined retrospectively using archival tissue from eight

patients who attended St Vincent's Hospital Dublin over a five year period from 1995 – 2005.

The Her2/neu, ER-1, and EGF-R1 expression was immunohistochemically detected on sections from formalin-fixed, paraffin-embedded tissues using monoclonal antibody staining with Trilogy antigen retrieval and The Vector Elite Detection system to visualise the antibody-antigen complex. Our controls were ER positive human breast tissue, HER positive human breast tissue and human placenta for erb-1.

With confirmation of low Estrogen receptor expression we explored the non estrogenic role of the most successful chemotherapeutic agent in oncology, Tamoxifen.

We analysed the anti-proliferative effects of Tamoxifen using colorimetric dimethyl-thiazol-diphenyltetrazolium bromide (MTT), pro-apoptotic effects were observed using flow cytometry. Tumour metastatic potential was investigated with a Matrigel invasion assay with tamoxifen and chemotactic agents VEGF and EGF. Anaplastic thyroid tumour cells are acutely sensitive to Tamoxifen. It induces apoptosis, decreases proliferation and the metastatic potential of ATC through blockade of a VEGF dependent mechanism. Tamoxifen now merits a phase I clinical trial as part of a multimodal chemotherapeutic agent treating ATC.

We then investigated the anti-neoplastic properties of 17-Allylamino-17-demethoxygeldanamycin (17-AAG) on CAL-62 and BHT-101 Anaplastic thyroid cell lines. The anti-proliferative effects were observed using colorimetric dimethyl-thiazol-diphenyltetrazolium bromide (MTT), and proapoptotic effects observed using flow cytometry (annexin V). Tumour metastatic potential was investigated with a Matrigel invasion assay with 17-AAG and chemotactic agents Epidermal Growth Factor and

Vascular Endothelial Growth Factor. We found 17 AAG to be a potent chemotherapeutic agent. It induces apoptosis, decreases proliferation and reduces the metastatic potential of the tumour cell through blockade of a Vascular Endothelial Growth Factor and Epidermal Growth Factor dependent, dual kinase mechanism. Heat shock protein 90 inhibitor 17 AAG is a promising new alternative treatment for ATC.

ATC remains a devastating disease for these unfortunate patients and their families. The National Institutes of Health lists seven treatment trials currently recruiting. Clinical trials are paramount in this rare devastating disease and success will have significant implications for the treatment of many other cancers. Multidisciplinary care and combination therapeutic strategies are required. Progress has been made understanding the cellular processes governing the mechanism of post malignant de-differentiation and the metastatic process. This has brought a variety of novel therapies of sufficient merit to conduct further phase trials. We believe this thesis offers fresh insight into all aspects of this disease which we hope, in part, may offer new clinical investigators a variety of options for oncology study.

Prologue

‘What is Past is Prologue’

William Shakespeare (1564–1616)

Hippocrates was born in 460 BC on the island of Cos. He believed man to be governed by the same laws as the cosmos, hence medicine must be an understanding, empirical and rational, of the workings of the body in its natural environment. Appeal to reason rather than to rules or to supernatural forces gives Hippocrates medicine its distinctiveness. It was also to win a name for being concerned more with observation and experience than with abstractions.

‘On the Nature of Man’, the body was viewed as stable until illness subverted it. Imbalance would produce illness if it resulted in undue concentration of fluid in a particular body zone. What was being kept in balance or upset were bodily fluids or *chymoi*, translated as ‘humours’. Two fluids were particularly associated with illness: bile and phlegm.

Although naturally present in the body, they seemed to flow immoderately in sickness. Black bile was considered an essential but mainly harmful humour. Visible in vomit and excreta, it was perhaps thought of as contributing to the dark hue of dried blood. Black bile completed a coherent, symmetrical grid in binary oppositions, and the four humours

– blood, yellow bile, black bile and phlegm- proved wonderfully versatile as an explanatory system.

They could be correlated to the four primary qualities- hot, dry, cold and wet; to the four seasons, to the four ages of man; infancy, youth, adulthood, and old age, to the four elements; air, fire, earth and water. They thus afforded a neat schema with vast explanatory potential.

Hippocrates employed the term *Karcinos* which is greek for crab. Presumably because the cancer pain resembles a crab's pinching or the creeping advancement of disease. He attributed tumour growth to an abnormal humoral accretion. Galen (born 129AD) regarded the disease as a species of inflammation.

These visionary scientists believed a tumour may arise from too much blood in the veins; or a flux of black bile mixed with blood producing a *Scirrhus*, a tumour which could convert to cancer. Hippocrates warned that surgery should not be used to treat deep or hidden cancers. This was endorsed by Galen.

Rhazes warned that surgery generally made matters worse unless the tumour was completely removed and the incision cauterised, and Pare confessed he had never seen cancer cured by the knife.

In 1622 Gasparo Aselli (1622) delineated the Lymphatic system. Lymph was increasingly blamed as carcinogenic rather than black bile. John Hunter maintained that under certain conditions coagulating lymph was the carcinogenic inflammatory factor.

The triumph of cell theory transformed the understanding of cancer. Virchow held that neoplasms developed from immature cells. By 1867 Klebs proposed that most cancers originated in epithelial tissues, however, it was Wilhelm Waldeyer who formed the basis of later theory. He maintained that cancer cells developed from normal tissue with cells multiplying by cell division and spread being lymphatic and haemogenic.

The Oncogenic Viral theory was championed by David Baltimore and Howard Temin who simultaneously published the discovery of Transcriptase. They believed a tumour causing virus enters a cell and RNA of the virus makes DNA copies of itself. This causes the host cell to become neoplastic. These theories remain controversial to this day.

i) Chemotherapy

The dream of chemotherapy dates back to Paul Ehrlich (1854-1964) who was a German bacteriologist who published 'Antimicrobial chemotherapy' where he described 'magic bullets', such as arsenic to treat syphilis. He won the Nobel Prize in 1908 for his work. Alexander Fleming, a Scotsman and bacteriologist at St Mary's hospital in London, (and a very distant relation to the author!), discovered the absence of bacterial growth in the area circumventing *Penicillium Notatum*.

Thus in true serendipitous fashion, the first identifiable antibiotic was born. He was knighted and won the Nobel Prize in 1945. Hopes were raised that a drug would be found to destroy cancer cells. In the 20's hopes were pinned on variants of the mustard gas prepared as a chemical warfare agent in the First World War. It was thought it would

prove a cytotoxic agent useful in cancer. As with other chemotherapeutic agents the problem with mustard gas was that it could not distinguish healthy from neoplastic cells. The drug produced a temporary improvement of symptoms but offered no long term cure. To date, no 'magic bullet' has been found for neoplastic cell destruction. It remains an elusive and enduring entity.

ii) Head and Neck Cancer

Head and neck cancers collectively comprise the eighth most common site for cancer in men and the sixteenth for women. There is great variety of tumour type and tumour outcome in Head and Neck cancer. Excluding skin, the breakdown of primary tumour location approximates the following: 40% oral cavity, 25% larynx, 15% oral and/or hypopharynx, 17% in the major salivary glands, and 13% in the remaining head and neck sites. The incidence of head and neck cancer is 2-3 times more common in men than in women and increases with age (American Cancer Society 2007).

In 2006 30,180 patients presented with thyroid cancer, 7,590 men and 22,590 women. In 2005 25,690 thyroid cases were reported; 6,500 men and 19,190 women. In 2004 23,600 cases reported, 6,950 men and 17,640 women. This is rapidly becoming a female dominated disease.

The prognosis of these head and neck heterogeneous group of tumours depends on the site. Tumours of the oral cavity survival rates can be dramatically improved with early detection and immediate treatment. For lip and oral cancer, if detected at its early stages, almost 80% of the patients survive five years or more. However, when diagnosed at the

advanced stages, the five-year survival rate drops to a mere 18%. Nose and sinus cancers of the nasal cavity often go undetected until they reach an advanced stage. If diagnosed at the early stages, the five-year survival rates are 60–70%. However, if cancers are more advanced, only 10–30% of the patients survive five years or more.

In cancer of the oropharynx, 60–80% of the patients survive five years or more if the cancer is detected in the early stages. As the cancer advances, the survival rate drops to 15–30%. Patients with nasopharyngeal cancer who are diagnosed with early stage cancers that have originated in the nasopharynx have an excellent chance of a complete cure (almost 95%). Unfortunately, most of the time, the patients are in an advanced stage at the time of initial diagnosis.

With the new chemotherapy drugs, the five-year survival rate has improved and 5–40% of the patients survive five years or longer. Small cancers of the larynx have an excellent five-year survival rate of 75–95%. However, as with most of the head and neck cancers, the survival rates drop dramatically as the cancer advances. Only 15–25% of the patients survive five years or more after being initially diagnosed with advanced laryngeal cancer. Regardless of the site of the primary tumour, the presence of a single lymph node in either the ipsilateral or contralateral side of the neck reduces the 5-year survival rate by 50%.

Treatment of head and neck cancer represents a significant challenge because of the poor prognosis, associated medical problems of this patient population, and adverse effects of treatment on patient function and appearance. Patients often are treated in a

multidisciplinary clinic. Treatment usually consists of some combination of surgery, chemotherapy, and radiation therapy. Indications for radiation therapy vary with tumour characteristics and location. Radiation or surgery can cure small tumours (T1-T2) in any location. The choice usually is made based on the functional aspects and potential complications of the two modalities. For example, T1 and T2 N0 tumours of the larynx can be treated with radiation with 90% cure rates for T1 lesions and 70% cure rates for T2 tumours.

Larynx preservation often is chosen for these smaller tumours since voice preservation obviously improves quality of life. The goal of any intervention is to completely treat the primary tumour and any draining lymphatics at risk for occult metastatic spread.

Combined therapy (surgery and radiation) usually is recommended for advanced (T3-T4) lesions. An expanding body of literature also supports chemotherapy with radiation therapy for advanced disease. If it is believed that the patient will survive the treatment, those with advanced disease will be treated with some sort of combined approach.

Two-thirds of patients present with locoregionally advanced disease and of these less than 30% are cured despite the frequent use of both treatment modalities. As many as 50% with advanced nodal disease may have micrometastatic systemic spread (Zbaeren P et al. 1987).

In the last two decades clinical research strategies have focused on the addition of chemotherapy to the armamentarium used against head and neck cancer. Traditional chemotherapy has been considered a standard therapy for patients who initially present

with systemic metastatic disease, who develop recurrence or have persistent disease after local therapy.

More recently chemotherapy can be considered a standard component, along with radiotherapy, of treatment for nasopharyngeal carcinomas, some laryngeal cancers and most unresectable cancers. Studies have demonstrated a benefit for patients receiving concomitant chemoradiotherapy either prophylactically or in the setting of residual disease following primary surgery, or for evidence of extracapsular spread (Haffty BG et al. 1997, Bachaud JM et al. 1996).

iii) Thyroid Cancer

The American Cancer Society estimates 37,340 new cases of Thyroid cancer in 2008 (American Cancer Society 2008). Thyroid cancer is broadly divided into differentiated and undifferentiated cancers and the five year survival is estimated at 97%.

Thyroid tumours represent a fascinating group of heterogeneous neoplasms. Papillary and Follicular carcinoma (well differentiated thyroid carcinomas) arise from the follicular epithelium and are the most common thyroid malignancies. The British Thyroid Association reports in 2001, data from Cancer Research UK showed 1,200 new cases in England and Wales, with a reported annual incidence for the UK of 3.5 per 100,000 women and 1.3 per 100,000 men. Thyroid cancer is the most common malignant endocrine tumour, but represents only about 1% of all malignancies (British Thyroid Association, 2007).

Thyroid cancers are followed by (according to traditional teaching) medullary thyroid carcinoma, anaplastic thyroid carcinoma and thyroid lymphoma. A rare form of thyroid cancer would be metastatic deposition from breast or colon disease.

The male to female ratio is approximately 2.5:1 however this may indeed be a conservative estimate. Presentation is largely 4th to 5th decades with median age of presentation 47 yrs of age. In a report of 15,700 patients in the USA, overall survival rates, corrected for age and sex, were 98% for papillary, 92% for follicular, 80% for medullary and 13% for anaplastic disease (Guilliland et al, 1997).

A thyroid nodule is the usual presenting feature of a thyroid neoplasm. Thyroid nodules are very common entities with 275,00 new nodules detected annually in The United States (Castro et al, 2000). An increasing number of incidental thyroid nodules are being identified due to the use of ultrasonography by the primary care physician.

The British Thyroid Association provides a useful criteria classification of patient referral into three groups; Patients with thyroid nodules who may be managed in primary care, patients who should be referred non-urgently and finally patients needing urgent referral. Cytological examination is the appropriate initial investigation with ultrasound. The size of the nodule that warrants Fine Needle Aspirate, FNA, is of constant debate. Many also argue Ultrasound should precede any aspiration due to tissue distortion post FNA. (Lalwani A. Lange/Mc Graw Hill 2004).

In general the majority of patients with well-differentiated thyroid carcinoma have a favourable long term prognosis with survival at ten years exceeding 80%. A number of

prognostic procedures have been developed to segregate patients with well differentiated thyroid carcinoma into a large group with a low risk of mortality and a small group with a high risk of mortality.

Key prognostic factors include age (the older the worst, key value 45 yrs), degree of invasiveness / extra thyroidal extension, presence of distant metastases, sex (males generally have a poor prognosis) and size. Staging methodologies that have been employed include TNM (tumour size, nodal metastatic status, distant metastatic status), AGES (age, grade, extent, size) or AMES (age, metastatic disease, extent and size).

Almost 90% of patients fit into the low risk category, with an overall mortality rate of 1-2%. About 10% of patients fit into the high risk category with a mortality rate of nearly 50%.

The epidemiology of this disease has changed dramatically in the last twenty years. (Appendix 1). The apparent increased incidence of this disease globally in particular in the United States with their SEER database results prompted us to investigate 'The Irish Experience'.

Thyroid cancer is also a controversial cancer as differentiated thyroid malignancies are implicated in the carcinogenesis of Anaplastic tumours. This phenomenon is termed the 'Post malignant de-differentiation'. The question is, does Anaplastic thyroid cancer arise *de novo* or is it part of this more sinister transformation process.

We will discuss this further in the introductory chapters however it would be largely agreed in the literature this process is real, however, the offending agents have not been identified and remain elusive. Improved understanding of this process will most certainly

open many doors of further question in oncology, not only in Thyroid but in allied cancers too.

iv) The American Joint Committee on Cancer Staging System (AJCC)

AJCC is used by Physicians and Surgeons around the world to facilitate the uniform description of cancer.

iv) a Stage Grouping

This table describes the Stage Groupings together as recommended for papillary or follicular, medullary and ATC.

Thyroid Group Staging			
Papillary or Follicular <i>Under 45 Yrs</i>			
Stage I	Any T	Any N	M0
Stage II	Any T	Any N	M1
Papillary or Follicular <i>45 Yrs and Older</i>			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1a	M0
	T2	N1a	M0
	T3	N1a	M0
Stage IVA	T4a	N0	M0
	T4a	N1a	M0
	T1	N1b	M0
	T2	N1b	M0
	T3	N1b	M0
	T4a	N1b	M0
Stage IV B	T4b	Any N	M0
Stage IVC	Any T	Any N	M1
Medullary Carcinoma			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1a	M0
	T2	N1a	M0
	T3	N1a	M0
Stage IV	T4a	N0	M0
	T4a	N1a	M0
	T1a	N1b	M0
	T2	N1b	M0
	T3	N1b	M0
	T4a	N1b	M0
Stage IVB	T4b	Any N	M0
Stage IVC	Any T	Any N	M1
ATC			
Stage IVA	T4a	Any N	M0
Stage IVB	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Explanation of thyroid cancer stage groupings.

Papillary thyroid cancer

Stage I papillary carcinoma is localized to the thyroid gland. In as many as 50% of cases, there are multifocal sites of papillary adenocarcinomas throughout the gland. Most papillary cancers have some follicular elements, and these may sometimes be more numerous than the papillary formations, but this does not change the prognosis. The 10-year survival rate is slightly better for patients younger than 40 years than for patients older than 40 years.

ATC

All Anaplastic carcinomas are considered Stage IV

Recent changes in the AJCC manual have resulted in the classification in ATC tumors changing to T4a, intrathyroidal anaplastic carcinoma- surgically resectable, and T4b extrathyroidal anaplastic carcinoma- surgically unresectable.

v) ATC

ATC remains the most aggressive disease in nature and the focus of this piece of work. We will now outline in the introductory chapter the main features of this disease and the therapeutic options currently available and accepted in the literature.

Chapter 1

Introduction

Anaplastic Thyroid Carcinoma

And research topics particular to this thesis

*‘If you wish to advance your knowledge of thyroid
cancer - study Anaplastic Thyroid Cancer ’*

Lancet

Editorial Comment 2005

**‘...There is a pressing need for novel chemotherapeutic strategies
in the treatment of anaplastic thyroid cancer...’**

1.1 Anaplastic Thyroid Carcinoma (ATC)

ATC is a rare neoplasm which holds a dismal prognosis. Fortunately the world wide incidence of ATC has decreased (Agrawi S et al 1996, Pasieka J et al 2003). Of the approximate 1500 deaths from thyroid cancer in 2007 in the United States, over half are directly due to the anaplastic variant (American Cancer Society 2007). According to our investigations the level of ATC in Ireland is 6.37% of all thyroid malignancies which is considerably higher than the American statistic of 1.27%.

ATC has a mean survival of 3-7 months after diagnosis, with a five year survival of 1% - 7.1% (Pasieka J et al 2003, Carcangiu ML et al 1985, Sugitani I et al 2001, Wiseman et al 2003, Venkatesh Y et al 1990, Demeter et al 1991, Passler C et al). Such is its biological aggression that tumour doubling time has been reported in one week (Ain et al 1999).

The histological types of thyroid cancer include papillary, follicular, hurtle, medullary and anaplastic carcinoma. Papillary, follicular and hurtle cell are considered well differentiated, however anaplastic is a poorly or undifferentiated carcinoma. The histological appearance is highly variable. There are three histologic patterns of ATC,

that is large cell, spindle cell and small cell variants. Gross appearance is a hard grey-white tumour. The tumour invades arteries and veins, occluding them while producing foci of infarction within itself.

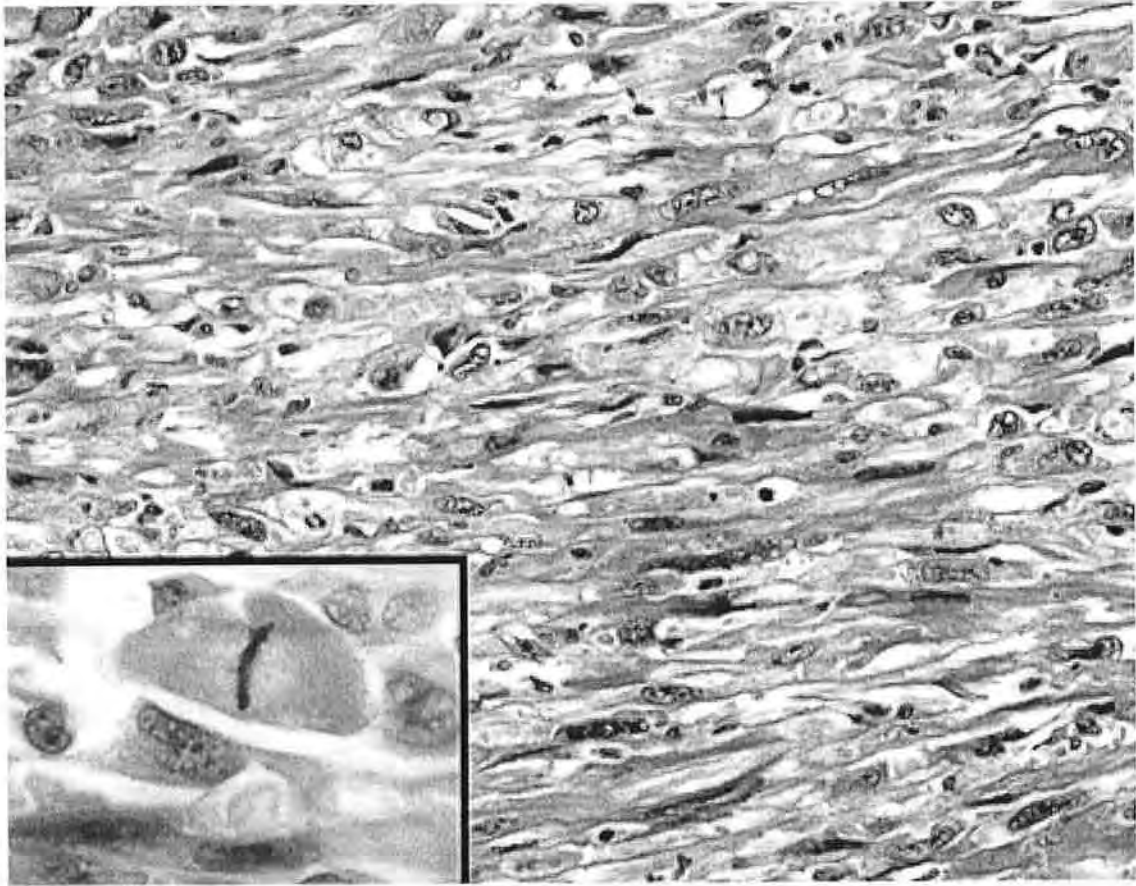


Fig 1.1 Photomicrograph of ATC Anaplastic (undifferentiated) thyroid carcinoma. The inset in the left lower corner shows a magnified view of a cell in metaphase of mitosis.

Source. Atlas of Genetics and Cytogenetics in Oncology 2009

Haematologyatlasgeneticsoncology.org/Tumors/Images/AnaCa

Tumors are in gross examination poorly defined, fleshy masses with areas of necrosis and hemorrhage. Microscopically they are composed of anaplastic cells with marked cytologic atypia and high mitotic activity.

Other patterns (e.g. angiomatoid, carcinosarcoma, lymphoepithelioma-like, adenosquamous) have been described. Undifferentiated (anaplastic) carcinoma of the thyroid must be differentiated from other high grade tumors with similar microscopic appearance originating from adjacent structures in the neck (e.g. larynx).

Sometimes this distinction is only possible on clinical/anatomical grounds. Immunohistochemically, undifferentiated thyroid carcinoma is generally negative for thyroglobulin and calcitonin. Pankeratin and epithelial membrane antigen (EMA) are positive in about one-half and one-third of cases, respectively. Thyroid transcription factor-1 (TTF-1) staining is present in 0-50% of cases.

Although there is variation within the microscopic classification of the disease, pathologic subtypes have identical clinical behaviours and have no differing prognostic significance. ATC can be considered as a single entity (Carcangiu et al 1985). Improved immunohistochemical techniques have revealed that the majority of small cell cancers are non-Hodgkin's lymphoma of the thyroid, "insular" variants of follicular, or medullary carcinoma (Kobayashi et al 1996, Mc Iver et al 2001).

1.2 Diagnosis

ATC presents in an elderly population, mostly six to seventh decades in life, marked by pain, dysphagia, hoarseness, and occasional dyspnoea with extensive local invasion of

surrounding tissues. 90% of patients have direct invasion of adjacent structures such as the peri-thyroid fat, trachea, esophagus, vasculature and muscles.

Distant foci of tumour are seen in 20% to 50% of patients. The most common sites of metastases are the lungs in 80%, bone in 6-15% and brain in 5-13% (Kobayashi et al 1996, Buzzoni et al 2003). Fine needle aspiration of a solitary thyroid nodule is the investigation of choice for suspicious neck lumps with ultrasound and CT scanning of the neck and chest. A solitary nodule over 3.0cm has a 30% incidence of malignancy (Shaha et al 1998).

Death is attributed to upper airway obstruction and suffocation in half of patients, and to a combination of complications of local and distant disease, therapy (or both) in the remainder (Jimin et al 2003). Although the mean survival is approximately 3-7 months, the most important prognostic factor appears to be the amount of disease present at the time of diagnosis (Sugitani et al 2001).

1.3 Anaplastic carcinogenesis

A progression model for thyroid carcinogenesis has not been defined. However, thyroid tissue undergoes molecular and genetic alterations provoking transformation from normal tissue to adenoma and from differentiated to undifferentiated carcinoma. It is generally accepted that this transformation proceeds through multiple discrete steps, as a single oncogenic mutation cannot induce malignant transformation on its own.

It is unclear if ATC can arise *de novo*. Furthermore there is no evidence to suggest malignant transformation is a structured and predictable process (Buzzoni et al 2003).

The transformation model of anaplastic tumourigenesis, or post-malignant transformation progression stems from pathologic observation of anaplastic tumours with a differentiated carcinoma component (Wiseman et al 2003, Cobin et al 2001, Rosen et al 1997, Nilsson et al 1998, Obara et al 1998, Kenneth et al 1999).

An association has been established between the aggressive histologic subtypes of papillary carcinoma (insular and tall cell) with ATC and with the hypothesis that they represent intermediate forms in the 'transformation' process (Rosen et al 1997, Kenneth et al 1999, Kim et al 1987, Sugino et al 2002). However, one institution's 50 year experience found no evidence of a differentiated carcinoma component within their anaplastic population (Mc Iver et al 2001).

Investigation of the DNA content of 11 ATC and adjacent differentiated carcinoma found all anaplastic tumours to be aneuploid, but only 7 of the 11 differentiated carcinomas were diploid thus lending further credence to the de novo hypothesis (Sugitani et al 2001).

Thyroid tumorigenesis is complex, involving several cell cycle regulators, oncogenes, growth hormones and cellular differentiation and adhesive compounds. Early stages of thyroid cancer development may be related to growth factor receptors or proto-oncogene activation (ret, met, ras) (Shimaoka et al 1985). Neoplastic expression of these genes is related to follicular (ras) and papillary (met, ret) carcinoma.

Mutation of tumour suppressor genes such as p53 or Rb (The retinoblastoma protein, abbreviated pRb or **Rb**) is a tumor suppressor protein that is dysfunctional in many types of cancer) is observed in poorly differentiated carcinoma. Mutations of p53 are

considered to be late events in the sequence of human carcinogenesis (Buzzoni et al 2003, Ahuja et al 1987, Nel et al 1985).

p53 is a tetrameric nuclear phosphoprotein transcription factor which is the product of the TP53 gene. The abrogation of function of the tumor-suppressor protein p53 as a result of mutation of its gene, TP53, is one of the most common genetic alterations in cancer cells. Disruptive TP53 mutations in tumor DNA are associated with reduced survival after surgical treatment of squamous-cell carcinoma of the head and neck (Poeta et al, 2007).

p53 is a tumour suppressor protein that acts in the nucleus to effect cell cycle arrest and apoptosis. p53 is mutated or absent in approximately half of all human cancers including lung, colon and breast (Kobayashi et al 1996, Besic et al 2001, Pierie et al 2002, Levendag et al 1993). 52% of ATC have shown TP53 or p53 mutation (Kim et al 2001). These mutations are rarely found in papillary or follicular carcinomas though genomic instability is present (Wiseman et al 2003, Ain et al 2000).

It is well documented that the re-introduction of wild-type p53 into ATC cells results in the induction of differentiation, inhibition of cellular proliferation, restoration of cellular responsiveness to physiologic stimuli (Thyroid Stimulating Hormone) and re-expression of thyroid peroxidase (Heron et al 2003, Simpson et al 1980). The introduction of TP53 gene therapy using adenovirus may serve as a significant adjunct to standard chemotherapy in management of this disease (Tennevall et al 2002).

Several strategies have been used to categorize *TP53* mutations, since different alterations have been observed to behave in different ways. The functional properties of each mutation may uniquely affect pathways for maintaining genomic integrity that involve p53. The biologic effects of *TP53* mutations may also be influenced by the presence or absence of the remaining wild-type allele and by the gain of function of some mutants.

TP53 mutations may be a useful stratification factor in prospective clinical trials. It would be clinically useful to determine whether *TP53* mutations are associated with a response to treatments that attack p53-specific pathways (Poeta et al, 2007).

1.4 MRP-1

Multidrug resistance (MDR) was first observed in experimental oncology in 1970 by Biedler and Riehm in a hamster model (Biedler et al, 1970). It has been extensively studied because one of the most serious problems in current cancer chemotherapy is intrinsic or acquired drug resistance. P-glycoprotein is a well-characterized ABC transporter of the Transporter associated with antigen processing (TAP) subfamily. P-gp is also called ABCB1, ATP-binding cassette sub-family B member 1, MDR1, and PGY1.

P-glycoprotein (P-gp) encoded by *mdr1* gene, is believed to play an important role in the mechanism of MDR, although the latter involves many factors. P-gp is a membrane ATPase that serves as an efflux pump for multiple anticancer agents. This protein has 12 transmembrane domains divided into two homologous halves, each of which includes an ATP-binding cassette domain that catalyzes ATP hydrolysis. A clinical relationship between overexpression of P-gp / *mdr1* with multiple drug resistance has been expressed while numerous researchers have demonstrated the direct relevancy.

The relationship between hormone and neoplasm is relatively complicated. It has been repeatedly demonstrated that estrogen has a close bearing on the carcinogenesis of its estrogen receptor (ER)-containing target organs such as uterus, vagina, breast and colon. ER expression in carcinomas was more extensive than in normal tissues indicating that high expression of ER and estrogen might play some role in differentiation.

Anti-estrogen tamoxifen (TAM) is one of the compounds that can modify multidrug resistance. It is widely used in the treatment of advanced breast cancers with a high response in tumors containing ER. Despite numerous *in vitro* studies and clinical trials of TAM having been conducted, it is necessary to investigate its effect *in vivo* or as *in vivo* replication and its relationship with ER status.

Multi drug resistance (MDR) was further described as a 170 kDa P-glycoprotein (P-gp) pump which can displace common cytotoxic drugs including anthracyclines, some vinca alkaloids and xenobiotics (Haigh et al 2001). Recent work on Non Steroidal Anti-Inflammatory Drugs (NSAIDS) show inhibition of the MRP-1 protein in a variety of cancer cell lines with a number of differing NSAIDS including sulindac, acetaminophen, mefenamic acid and Indomethacin (Segev et al 2003, Ibanez et al 1966). The MRP-1

protein is expressed in anaplastic thyroid cancer (Rodriguez et al 2000, Nishiyama et al 1972) and further research is required to investigate its inhibition offering synergistic chemotherapeutic potential.

1.5 Current therapeutic strategies

1.5.1 Surgery

It is agreed in the literature that combination therapeutic strategies will afford us an improvement in survival statistics (Tan et al 1995, Spires et al 1988, Lam et al 2000, Saunders et al 1999, Wallin et al 1989, Moretti et al 2000). The extent of surgical resection of ATC remains controversial. The locally invasive nature of ATC often results in incomplete resections (Fagin et al 1993). Furthermore, as up to 50% of patients on presentation have distant metastases (Kobayashi et al 1996, Buzzoni et al 2003), palliative resection may be the only viable option.

Total thyroidectomy is only justified if mediastinal and cervical disease can be completely removed with limited morbidity (Lowe et al 1995, Nagayama et al 2000). The role of extended resection including pharyngoesophagectomy and total laryngectomy is open for debate (Saunders et al 1999, Steels et al 2001). No effective surgical treatment is available for distant metastases of ATC.

The Mayo clinic recently published an ominous account of the last fifty years of surgical experience with ATC. They found no survival advantage between radical and marginal resection (Mc Iver et al 2001). However a survival advantage (2 vs 6 months) has been reported with macroscopic clearance (Campo et al 1991).

The integration of chemotherapy into the radiotherapy and surgical formula also improved survival. The order of therapeutic strategy is now the focus of research and debate.(Spires et al 1988, Marks et al 1994)

1.5.2 Surgery / Radiotherapy

Results of retrospective series of treatment of ATC with radiotherapy alone, or post surgery, are conflicting, though appear to suggest that higher doses of radiotherapy (RT) are associated with improved survival.

A Dutch review (Stoler et al 2002) of 67 ATC patients revealed a survival advantage relating to extent of surgery and radiation dose. Surgery was performed in 44 of 67 patients, with 12 complete resections. The 6-month and 1- and 3-year survival rates were 92%, 92%, and 83% after complete resection; 53%, 35%, and 0% after debulking; and 22%, 4%, and 0% after no resection, respectively ($P < .0001$). A radiation dose of >45 Gy improved survival as compared with a lower dose ($P = .02$). Multivariate analysis showed that age ≤ 70 years, absence of dyspnoea or dysphagia at presentation, a tumor size ≤ 5 cm, and any surgical resection improved survival ($P < .05$). Of course the extent of surgery relating to a survival advantage is not confirmed by other retrospective reviews.

Levendag et al in 1993 reported on 51 patients treated with palliative RT with or without chemotherapy. This showed similar results indicating that RT in excess of 30 Gy improved survival (3.3 v 0.6 months for those < 30 Gy). A survival advantage was also observed for those who completely responded to RT over those with a partial response (7.5 v 1.6 months), a common strain in these reviews. However, some patients who achieve local control will die shortly after treatment due to metastatic disease.

A series from the Beatson Oncology Centre in Glasgow (Lowe et al 1995), did not show a survival advantage to RT, despite an 80% response rate.

1.5.3 Chemotherapy

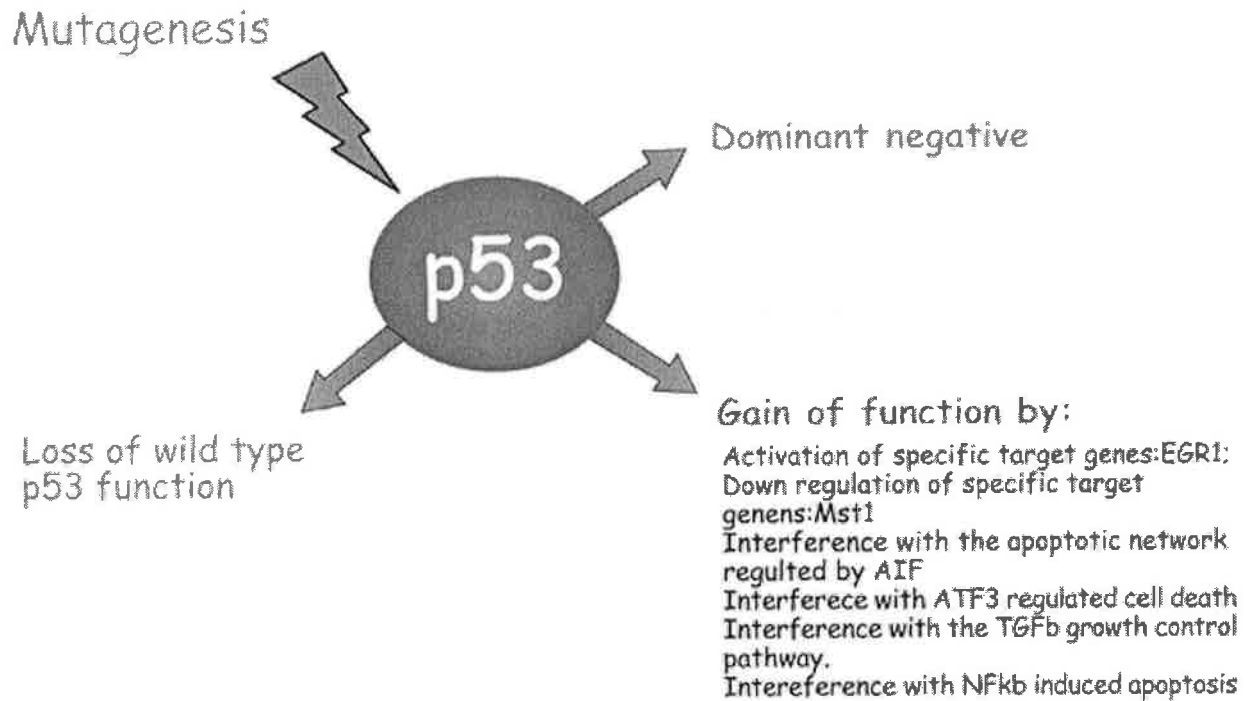
The majority of ATC patients develop metastases during their illness. Hence there is clearly an essential role for systemic chemotherapy (C). Doxorubicin has been the most commonly reported drug, but monotherapy has been shown to have less than 20% response rate, with no complete responses.

The addition of cisplatin to doxorubicin has modestly improved response rates (Lowe et al 2005, Nagayama et al 2000). A phase II trial (Ain et al 2000) from the Veterans Affairs Medical Centre, Lexington, Kentucky reported a 53% response rate with the single agent paclitaxel. Twenty patients with persistent or metastatic ATC were treated with 96-hour continuous infusion paclitaxel every 3 weeks for 1 to 6 cycles. This regimen was well tolerated though grade 3 peripheral neuropathy was experienced with some schedules. Median survival of responders was 32 weeks, though that of the non-responders was only 7 weeks. There was one complete response.

Yeung et al 2000 at M. D. Anderson found that the addition of manumycin (a farnesyl: protein transferase inhibitor) in vitro enhanced Paclitaxel cytotoxicity.

German investigators, Voight et al 2000, have researched cisplatin and gemcitabine alone or in combination in ATC cell lines. Gemcitabine monotherapy showed promising cytostatic activity, enhanced by the addition of cisplatin. Their interaction was schedule and dose dependent, favouring a regimen in which gemcitabine is followed by cisplatin. DNA-synthesis inhibition and S phase arrest may be important determinants for this drug interaction.

Oncogenic function of mutant p53 proteins



'p53 in Normal and Tumor Cells'

The Norman and Helen Asher Chair of Cancer Research

Department of Molecular Cell Biology

Weizmann Institute of Science

p53 mutations are thought to be associated with reduced chemosensitivity and radiosensitivity (Yeung et al 2000, Voigt et al 2000). p53 mutation lies at the heart of the anaplastic tumorigenesis debate. Either the mutation allows for accelerated genomic instability or loss of wild type p53 results in growth, angiogenesis and the development of the anaplastic undifferentiation, hence anaplastic tumorigenesis (Yeung et al 2000, Combretastatin et al National Cancer Institute).

Data from a National Cancer Institute trial (ICC 2302) of induction doxorubicin/cisplatin followed by combretastatin A4 Phosphate and RT for newly diagnosed regionally advanced ATC is accruing. Combretastatin A4 phosphate (CA4P) represents the lead compound in a group of novel tubulin depolymerising agents being developed as vascular targeting agents. Preclinical studies have shown that CA4P induces blood flow reductions and subsequent tumour cell death in a variety of preclinical models.

1.5.4 Surgery / Chemo-Radiotherapy

Heron et al (2003) at the University of Pittsburgh demonstrated a survival advantage using hyperfractionated RT and C compared to conventional RT alone. Thirty two ATC patients treated over a period of 5 decades were analysed.

A variety of radiotherapy techniques was used. Chemotherapy consisted of doxorubicin, paclitaxel, vincristine, or cisplatin. Among patients with ATC surgery, hyperfractionated radiotherapy in conjunction with chemotherapy was associated with better survival but not progression-free survival compared to conventional radiotherapy.

Doxorubicin enhances RT toxicity, and it is unusual to instigate concomitant therapy at standard doses. Simpson et al (1980) devised a protocol of hyperfractionated radiotherapy, administering a small number of large radiation fractions (350 to 800 rads) with concomitant doxorubicin. Local response was good, though toxicity was unacceptable. Two patients died of spinal cord necrosis and a third of pneumonitis.

Kim and Leeper (1987) at Memorial Sloan-Kettering modified Simpson's approach. Nineteen patients with anaplastic giant and spindle cell carcinoma of the thyroid received Doxorubicin (10 mg/m², a low dose) once weekly before hyperfractionated RT.

Radiation therapy was carried out with a fractional dose of 1.6 Gy per treatment twice a day for 3 days per week to a total dose of 57.6 Gy in 40 days. Despite an initial complete tumor response rate of 84%, median survival was only 1 year. Unlike Simpson's data, no disproportionately enhanced normal tissue morbidity was seen.

1.5.5 Neo-Adjuvant Vs. Adjuvant Chemo-Radiotherapy

Between 1984 and 1999, 55 consecutive ATC patients at the Lund University Hospital in Sweden were prospectively treated with hyperfractionated RT, doxorubicin, and surgery when feasible. RT was delivered at 1.0 to 1.6 Gy twice a day, to a total dose of 46 Gy. Some patients received all RT prior to surgery, and some as a split course, 30 Gy pre-op, and 15 Gy post-operatively. Surgery was performed in 40 patients. No patient failed to complete the protocol due to toxicity. Death was attributed to local failure in only 13 cases (24%). Five patients (9%) lived more than 2 years. Results were best for the entire pre-operative 1.6 Gy twice a day regimen. Seventeen out of 22 did not recur locally. Of those who proceeded to surgery in this group, none failed locally.

One hundred and sixty two patients with ATC treated at the Institute of Oncology, Ljubljana have been studied. There was no difference in 1 year survival between patients who underwent surgical resection followed by CRT, compared to neo-adjuvant C, RT, or CRT. It is interesting to note that the primary C or RT group was composed of older patients, with faster growing, larger tumours. Despite this, survival was the same, 50% were alive in one year.

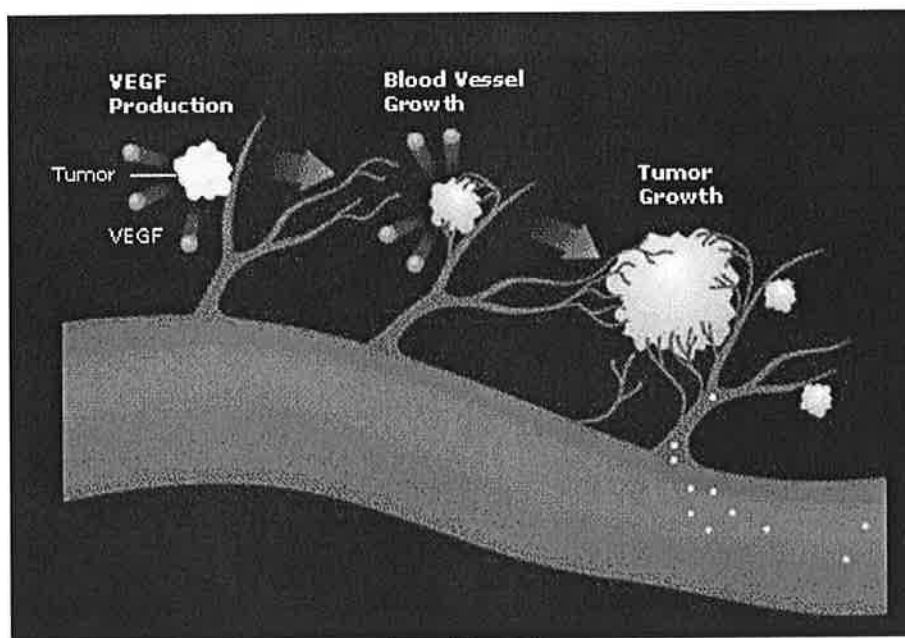
Anaplastic thyroid carcinoma remains a highly aggressive lethal disease whose origins remain controversial. Does ATC arise *de novo* or is it part of a de-differentiation, post

malignant transformation? The cellular processes governing these mechanisms remain unknown and with continued research offer future therapeutic potential.

Despite reports of adequate local control, survival with multimodal therapy remains poor. A common strain in combination therapy reviews is that a small cohort of complete responders have significant survivals, thus emphasizing the need to treat appropriate patients adequately. Neo-adjuvant chemoradiotherapy may be superior to adjuvant. Systemic chemotherapy outside multimodality therapy should be confined to clinical trials.

1.6 Angiogenesis & Vascular Endothelial growth factor

1.6.1 Angiogenesis



Source: Genentech.

(Genetech website, 2008 www.genetech.com)

New vasculature is formed by a combination of two mechanisms, vasculogenesis and angiogenesis (Noden D.M. 1989). Vasculogenesis is the process whereby angioblast cells

in the mesoderm differentiate into endothelial cells and give rise to primitive blood vessels (Risau W et al. 1995). Angiogenesis is a process through which new blood vessels develop from pre-existing vessels such as capillaries and post capillary venules (Varner J et al. 1996, Noden DM. 1989). Within the growing embryo the vasculature is formed by a combination of vasculogenesis and angiogenesis (Noden et al. 1989). In adults angiogenesis is the sole method for new blood vessel formation.

1.6.2 Physiological and pathological angiogenesis

Angiogenesis occurs in a highly regulated fashion during normal physiological events.

It remains inhibited, apart from tightly controlled physiological situations such as the development of the ovarian follicle, corpus luteum and endometrium in the menstruating female (Reynolds LP et al. 1992) and during wound healing (Arbiser JL. 1996). Revascularisation of tissue in a wound bed following injury is essential for the delivery of nutrients and immune cells and removal of debris and is accomplished by angiogenesis (Arbiser et al. 1996). Angiogenesis is also required for the development of healing bones (Winet et al. 1996). Under these circumstances, angiogenesis is briefly turned on and then completely shut down. Aberrant angiogenesis can occur in a number of pathological states other than tumour growth. It is a vital component of certain disease conditions such as psoriasis (Malhotra R et al. 1989), rheumatoid arthritis (Koch AE. 1998), diabetic retinopathy (Folkman J. 1992), atherosclerosis and chronic inflammation. Angiogenesis is now recognized to be essential for the growth and metastasis of solid tumours (Folkman J. 1971 & 1990, Gasparini G. 1994). Growth of a tumour beyond 2-3 mm³ requires development of a microvessel network to facilitate delivery of nutrients and oxygen, and removal of catabolites.

Numerous studies have documented the importance of angiogenesis for tumour growth. Gimbrone and colleagues implanted a rabbit epithelioma in the anterior chamber of the eye, where it is impossible to attract new blood vessels, and found that while it remained viable over a 34-day period, its size did not increase beyond 1mm^3 . However, when the same tumour was then reimplanted in the iris, where the induction of new vessel growth is possible, it reached 16,000 times its original volume within 14 days (Gimbrone MA et al. 1972). A rat sarcoma, implanted on the chick chorio-allantoic membrane (CAM) does not show any evidence of growth during the first 72 hours, when there is no evident blood supply. An almost exponential increase in growth rate is observed, however, within 24 hours of the ingrowth of new vessels (Ingber DE et al. 1986). Holmgren has shown that, prior to the onset of angiogenesis, these 2mm^3 islets of tumour are in a steady state of proliferation balanced by apoptosis (Holmgren L et al. 1995).

1.6.3 Angiogenic progression

The target vessels for angiogenic factors are the post capillary venules and small terminal venules (Guvakova and Surmacz. 1997). These vessels are comprised of endothelial cells that lie upon a basal lamina surrounded by a discontinuous layer of pericytes and smooth muscle cells embedded in the extracellular matrix. The morphology of the endothelial cells is altered in response to angiogenic factors. There is an increase in the amount of endoplasmic reticulum, Golgi apparatus and mitochondria and the endothelial cells form protrusions on the abluminal side (Fox et al. 1996). On the other hand, newly formed capillaries are composed of two cell types, endothelial cells and pericytes. These two

cells have the capacity to produce entire capillary networks. Following the transduction of signals that promote differentiation in vivo, angiogenesis progresses in four stages: activation of endothelial cells, proliferation, migration and lumen formation (Cockerill GW et al,1995). Cytokine release is provoked by factors endogenous and exogenous to tumour cells, including local environmental factors such as hypoxia. Quiescent endothelial cells are activated by the release of cytokines from host and tumour cells (stage 1). Committed cells proliferate (stage 2), and then migrate along a fibrin skeleton towards the source of the angiogenic stimulus to form cords of aligned cells (stage 3). Finally, the vascular sprout forms a lumen and the cells exit the cell cycle to a resting phase. Development of a patent lumen (stage 4) occurs through coalescence of intracellular vacuoles and is facilitated by cell-to-cell adhesive contact (Varner J et al.1996).

The capillary sprouts in tumours are 'leaky' as proliferating capillaries have incomplete basement membranes. In addition, vascular endothelial growth factor (VEGF; previously known as vascular permeability factor) increases permeability through the development of a series of interconnected cytoplasmic vesicles and vacuoles (known as vesical-vacuolar organelles) that maintain contact with both the luminal and abluminal surface (Senger DR et al. 1993, Cornali E et al. 1996). In normal tissues this may play a regulatory role in controlling baseline microvascular permeability (Berse B et al. 1992), and in tumour microvessels this feature has been linked to malignant exudates and ascites (Nagy JA et al. 1989, Donovan D et al. 1997).

Tumour vessels also differ from those of normal tissue, in terms of permeability, cellular composition, stability and regulation of growth (Folkman J et al. 1976). This suggests

that antigenic differences may exist which may be exploited in specifically targeting tumour vasculature (Denekamp J. 1993, Fan TPD et al. 1995). A number of antigens preferentially expressed in tumour vasculature have now been identified such as VEGF and Tie Receptor kinase. Even after all these events have taken place there is a constant remodelling of formed vessels as well as the recruitment of new vessels so that the tumour vasculature is highly unstable (Fox et al. 1996). Endothelial cells are highly heterogeneous (McCarthy et al. 1991) and the morphology of the tumour vasculature is highly variable between tumours of different sites (Roberts et al. 1998).

1.6.4 The angiogenic ‘switch’

Solid tumours express genes that code for angiogenic stimulators suggesting that the ability to form new vessels is part of the malignant phenotype (Risau W. 1995, Folkman J et al. 1996, Bouck N et al. 1996). There is little doubt that an angiogenic phenotype develops during tumour progression, and that the development of a blood supply is vital in the escape from dormancy. Studies of oncogene expressing mouse pancreatic islet cells show that whilst these cells readily become hyperplastic, only a few become angiogenic, and this angiogenic activity is necessary for, and precedes, tumour formation (Folkman J et al. 1989). In another mouse model, basal keratinocytes were transformed into a squamous cell carcinoma by the human papilloma virus type 16 oncogene and tumour progression was only seen after sudden onset of angiogenesis that had been quiescent until that point (Coussens et al. 1996).

Examination of sections from cervical smears has demonstrated that angiogenesis precedes late stage disease but is absent in the earlier stages of this cancer (Guidi et al. 1995). This demonstrated, within these studies that increased tumour cell proliferation is

not sufficient for the development of angiogenesis but that another key step, the acquisition of an angiogenic phenotype is also required. This has led to the development of the concept that tumours must activate an angiogenic “switch” before tumour growth can progress to the formation of a clinically relevant tumour (Hanahan and Folkman. 1996).

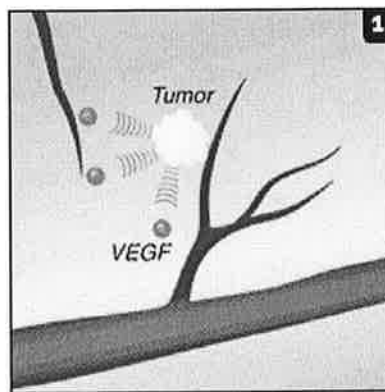
Under normal physiological conditions angiogenesis is regulated by the release of antiangiogenic factors (Quinn et al. 1993). Malignant transformation is a cumulative process requiring loss of control of the cell cycle and a shift in the balance of proangiogenic and antiangiogenic factors. Induction of angiogenesis is a local event, which is specific to activated hyperplastic cells and which precedes overt tumour formation (Folkman J et al. 1989). For example, angiogenesis is a marker of premalignant transformation in benign breast disease (Brem SS et al. 1978 & 1977). Vascular endothelial cells in malignant breast tumours express tissue factor, a potent procoagulant, whereas vascular endothelial cells in benign tumours do not (Contrino J et al. 1996). Cell proliferation and malignant transformation represent a switch to an angiogenic phenotype, but only in the latter is control of the cell cycle lost. Furthermore, only those hyperplastic cells that acquire angiogenic capacity undergo malignant transformation (Folkman J et al. 1989, Brem SS et al. 1977).

1.6.5 Vascular Endothelial Growth Factor

The literature now indicates that there may be a tissue specific relevance for Vascular Endothelial Growth Factor (VEGF) in the development of cancer. (Huang et al, 2001)

VEGF plays an important role in thyroid carcinogenesis and cancer progression. The potential implications of VEGF in the progression of thyroid neoplasms appear to be

diverse for each histologic subtype. Viglietto et al first demonstrated that expression of VEGF is positively associated with tumorigenic potential of thyroid cancer cell lines. (Viglietto et al, 1995). VEGF protein expression was higher in cancer cell lines and a few primary tumours than in primary cultures of normal thyroid cancer cell lines. Those tumours with high VEGF content had a high index of cell proliferation. VEGF is implicated in angiogenesis and metastases as the initiation of a vascular phase marks a period of accelerated growth, local invasion and ultimately metastases of thyroid neoplasms.



(Genetech 2008)

The sequence of events which lead to the development of a vascularising tumour phenotype are complex and involve many growth factor dependent mechanisms, including those dependent on interactions with various angiogenic growth factors. A variety of cytokines have previously been identified as potential positive regulators of angiogenesis. VEGF is the most potent directly acting angiogenic protein known (Mukhopadhyay D et al. 1995). It is a diffusible endothelial cell-specific mitogen and angiogenic factor, which also increases vascular permeability (Ferrara N. 1995, Marme D. 1996). VEGF is a 34-42 kDa, basic, heparin-binding, homodimeric glycoprotein that

is heat and acid stable. The human VEGF gene has been localised to chromosome 6p12 (Wei MH et al.1996). Alternative splicing of mRNA is responsible for the existence of VEGF in four different isoforms, VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆, depending on the number of amino acids contained in each isoform. VEGF₁₆₅ (the predominant isoform) and VEGF₁₂₁ are secreted, whilst VEGF₁₈₉ and VEGF₂₀₆ have a high affinity for heparin and are almost completely sequestered bound to heparin-containing proteoglycans in the extracellular matrix (Connolly DT. 1991, Schott RJ et al. 1993, Ferrara N et al. 1993, Senger DR et al. 1993). To date, there is little information on which isoforms are expressed within tumours. Most studies have either measured total VEGF mRNA or VEGF₁₆₅ protein.

In situ hybridisation studies have shown that VEGF mRNA is markedly up-regulated in virtually all human tumours examined thus far, including kidney and bladder tumours (Brown L et al. 1993), breast cancer (Brown L et al. 1995), ovarian carcinoma (Olsen TA et al. 1994) and gastrointestinal malignancies (Brown L et al. 1993) as well as intracranial tumours (Phillips et al. 1993). The essential role of VEGF in blood vessel formation can be seen even in the early stages of embryonic development. The formation of blood vessels was abnormal in mice embryos that had a heterozygous deficiency for the VEGF gene (VEGF +/-) (Carmeliet et al. 1996). Mice embryos that had a homozygous deficiency for the VEGF receptors VEGFR-1 and VEGFR-2 also died in utero after approximately 9 days (Fong et al. 1995, Shalaby et al. 1995). There were no organised blood vessels within the embryo or yolk sac at any stage of development. Several recent studies have demonstrated the vital role played by VEGF in the development of the tumour vasculature. A study performed in nude mice showed that expression of VEGF₁₂₁

or VEGF₁₆₅ conferred the ability to form vascularised tumours on a previously non-tumourigenic Chinese hamster ovary cell line (Ferrara N et al. 1993). Mouse embryonic stem cells with homozygous deficiencies for VEGF have a dramatically reduced ability to form tumours in nude mice compared to normal stem cells and the tumours have a significantly reduced number of blood vessels (Ferrara et al. 1996). Administration of a monoclonal antibody against VEGF was found to inhibit the growth of a variety of tumours in mice including human rhabdomyosarcoma, glioblastoma and fibrosarcoma (Kim et al. 1993, Asano et al. 1995, Borgstrom et al. 1996). More recently, the use of intravital videomicroscopy techniques has demonstrated that anti-VEGF antibodies block tumour angiogenesis (Borgstrom P et al. 1996).

VEGF promotes angiogenesis via a number of different mechanisms. It has been shown to induce the expression of the serine proteases urokinase-type (uPA) and tissue-type (tPA) plasminogen activators (PA) and PA inhibitor (PAI-1) in bovine microvascular endothelial cells (PepperMS et al. 1991). Human umbilical vascular endothelial cells express the metalloproteinase interstitial collagenase in response to VEGF (Unemori EN et al. 1992). These actions promote a pro-degradative environment that facilitates the migration of endothelial cells.

VEGF has a potent permeability enhancing effect on vessels and is also known as vascular permeability factor (VPF) (Senger DR et al. 1983). This is achieved via a direct effect on the endothelium and not by means of mast cell degranulation (Koch AE. 1994). VEGF has also been shown to induce vasodilatation in vitro in a dose dependent manner most likely via nitric oxide production (Kud D et al. 1993, Yang R et al. 1996) and VEGF is chemotactic for endothelial cells (Yoshida A et al 1996).

Tumour-derived VEGF plays an important role in the paracrine stimulation of angiogenesis but it also appears to have an autocrine stimulatory effect on tumour cells (Liu B et al. 1995), particularly in response to hypoxia (Namiki A et al. 1995). Hypoxia stimulates angiogenesis in a number of sites, including endothelial cells, retinal pericytes (Takagi H et al. 1996), myocardium (Ware JA et al. 1997) and solid tumours (Shweiki D et al. 1992). VEGF activity is potentiated by oxygen deprivation (Minchenko A et al. 1994, Levy AP et al. 1995, Grone HJ et al. 1995), mediated in part by adenosine (Takagi H et al. 1996, Hashimoto E et al. 1994) through upregulation of the VEGF endothelial cell receptor KDR, which stands for Kinase domain region and is the VEGF Receptor-2. (Brogi E et al. 1996). Wild-type p53, a tumour suppressor gene, inhibits proliferation of both normal and transformed cells (Finlay CA et al. 1989, Levine AJ et al. 1991), and also regulates VEGF production. Mutations in this gene abolish such control. There appears to be two regulatory VEGF pathways: an oncogenic one (v-src) that enhances VEGF production and a tumour suppressor (p53) signal that suppresses VEGF (Mukhopadhyay D et al. 1995). Mutations of the ras oncogene cause VEGF upregulation in a colon cancer model with resulting increased angiogenesis (Rak J et al. 1995). H-ras oncogene mutation also activates angiogenesis through upregulation of VEGF and matrix metalloproteinase (MMP) bioactivity, while downregulating activity of tissue inhibitors of MMP (Arbiser JL et al. 1997). Several cytokines or growth factors also have a role in the upregulation of VEGF mRNA expression or in the induction of the release of VEGF protein. These include Epidermal Growth Factor (EGF), Transforming Growth factor Beta- β 1 (TGF-1) (Frank S et al. 1995) and Interleukin-1 (IL-1) (Li J et al. 1995).

Oncogenic mutations or *ras*-amplifications have been shown to lead to VEGF up-regulation in transfected cells (Rak J et al. 1995).

VEGF mRNA is expressed in tumour, but not endothelial cells. Tumour associated endothelial cells display an up-regulation of mRNA for the VEGFR-1 and VEGFR-2 receptors, when compared to the vasculature of the surrounding tumour free tissue (Brown LF et al. 1995 & 1993, Plate KH et al. 1992). High affinity VEGF binding sites are localised to the vascular endothelium of large or small vessels. The two main receptors are the VEGFR-1 (formerly known as Flt-1, fms-like tyrosine kinase)(Shibuya M et al. 1990) and VEGFR-2 (previously KDR, Kinase domain region) (Terman BI et al 1991) proteins. Of the two, VEGFR-1 binds with the highest affinity to rhVEGF₁₆₅.

More recently, additional non-angiogenic effects of VEGF protein have been identified. These effects seem to have a tumour promoting effect as well, and add weight to the argument for the administration of an anti-VEGF agent in the treatment of cancer. Gabrilovich and colleagues found that VEGF inhibited maturation of dendritic cells, which are important antigen-presenting cells (Gabrilovich Di et al. 1999). Thus, prolonged exposure of the immune system to high levels of circulating VEGF may allow tumours to avoid induction of an immune response (Salven P. 1998). Katoh and colleagues report that VEGF may suppress apoptotic cell death in haematopoietic cells and thus may confer a degree of resistance of leukaemia cells to ionising radiation (Katoh O et al. 1995). The gene possibly responsible for this, ZK7, has now been cloned (Kuramoto K et al. 2000). The same authors have also demonstrated that VEGF inhibits apoptotic cell death induced by exposure to the chemotherapeutic agents doxorubicin and etoposide (Katoh O 1998).

Nor et al demonstrated that VEGF acted as a survival factor for endothelial cells, preventing apoptosis by inducing Bcl-2 expression (Nor J et al, 1999). Following this Pidgeon et al (2001) found that VEGF upregulated Bcl-2 expression and anti-VEGF antibodies reduced Bcl-2 expression in two breast carcinoma cell lines. The levels of tumour cell apoptosis reflected these alterations in Bcl-2 expression. VEGF resulted in reduced tumour cell apoptosis, whereas its inhibition with anti-VEGF neutralizing antibodies induced apoptosis directly in tumour cells. Therefore, in addition to its role in angiogenesis and vessel permeability, VEGF acts as a survival factor for tumour cells, inducing Bcl-2 expression and inhibiting tumour cell apoptosis.

VEGF appears to play a role in suppressing the immune surveillance mechanism and in promoting resistance of tumours to standard cytotoxic treatment. Blocking the effect of this cytokine in the tumour patient may well prove a worthwhile addition to the clinicians' armamentarium. We highlight the metastatic role of VEGF in our investigations with Tamoxifen.

1.7 Epidermal Growth Factor

One of the characteristics of many cancers is an abnormal increase in the activity of epidermal growth factor receptors. These receptors are found in the cell membranes. When activated, these they initiate a signaling process that regulates cell growth Tumors that show overexpression of epidermal growth factor receptors include cancers of the lung (non-small cell), colon and rectum, breast, pancreas, prostate, ovary, head and neck, esophagus, brain (glioblastoma) and thyroid cancer, especially the ATC variant.

This increased activity leads to uncontrolled cell growth, with decreased apoptosis and increased angiogenesis. The overexpression of receptors leads to activation of other genes that promote cancer growth through such means as invasion and metastasis, and resistance to chemotherapy and radiotherapy.

The Epidermal Growth factor receptor (EGFR) is a cell membrane receptor that plays a key role in cancer development and progression. Increasing evidence has shown that the over-expression of EGFR closely correlates with advanced tumour stage and metastasis, and poor clinical outcome in many human cancers including breast, cervix, lung, bladder and head and neck. (Iihara et al 1993, Hu et al 1997, Grandis et al, 1998, Brabender et al, 2001, Arteaga and Truica, 2004)

Ligand binding to EGFR induces dimerisation of the receptor. Homo and/or heterodimerisation of EGFR activates intrinsic tyrosine kinase, leading to receptor autophosphorylation, then activates a number of intracellular signal transducing elements. Phosphatidylinositol-3-kinase, protein kinase B/AKT (akt), a small G-protein (ras) the ras GTPase-activating protein, extracellular signal-regulated kinase (ERK) 1/2, Src family kinase, and STATs mediated pathway are known downstream effectors of the EGFR. A cell proliferating signal from the activated EGFR reaches ERK1/2 through ras/raf activation, and is transmitted to intranucleus cell proliferation signals. Akt also transmits the signal from activated EGFR, to inhibit apoptosis (Raymond et al, 2000).

EGFR targeted therapies have now attracted increasing attention in the literature. There are many kinds including antireceptor monoclonal antibodies, antiligand monoclonal

antibodies, ligand-toxin conjugates, scFv-toxin conjugates, ligand-genistein conjugates and tyrosine kinase inhibitors (Iressa: ZD 1839). Anti-tumour activity of Gefitinib has demonstrated significant anti-tumour activity at phase II trials in advanced non small cell lung cancer and is now approved worldwide. In this work we study the expression of Epidermal Growth Factor in Anaplastic Thyroid cell lines. We also examine the response these cells have to the tyrosine kinase inhibitor 17-(allylamino) 17-demethoxygeldanamycin (17AAG).

1.8 Heat Shock Proteins

1.8.1 Molecular Chaperones

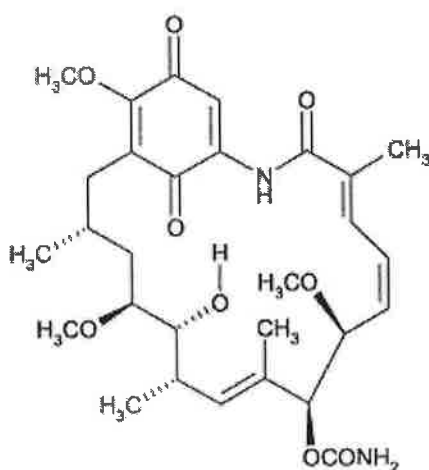
Molecular chaperones are a class of proteins that guide the normal folding, intracellular disposition and proteolytic turnover of key regulators of cell growth and survival. This function is subverted during carcinogenesis to allow malignant transformation.

Most heat-shock proteins (HSPs) levels of intracellular expression increase in response to protein-denaturing stressors, for example a change in temperature, as an evolutionarily conserved response to restore the normal protein-folding environment and maintain cell survival. HSP90 acts as a biochemical buffer allowing mutant proteins to retain or even gain function while allowing cancer cells to tolerate the unbalanced signalling that such oncoproteins create. Specific inhibitors of HSP90 have been identified that redirect its chaperoning activity and decrease cellular levels of many cancer-related client proteins that depend on this molecular chaperone. The use of HSP90 inhibitors is invaluable at a basic level in probing the complex cellular functions of this chaperone. The best way to

exploit the novel mechanism of action of HSP90 inhibitors for anticancer therapy remains to be defined, but probably involves combination with conventional chemotherapeutic agents or other molecularly targeted agents. We observe the application of this drug and its effects using a wide variety of laboratory tests with the in vitro Anaplastic Cancer cell lines in this thesis.

1.9 17-(allylamino)-17-demethoxygeldanamycin

Improved insight into molecular, genetic, and biochemical changes occurring during the process of carcinogenesis have changed the focus of drug development from empirical therapy towards therapies acting at specific molecular targets which are responsible for the neoplastic phenotype.



Molecular Structure of 17 AAG

www.invivogen.com/images

Several chemotherapeutic agents that do not depend upon the p53 pathway for their cytotoxic mechanisms have been introduced as novel anticancer drugs for tumours with

mutant p53. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is a derivative of Geldanamycin, a benzoquinone ansamycin antibiotic which binds to Hsp90 (heat shock protein 90) and alters its function (Blagosklonny et al 1995, Gorski et al 1999, Egorin et al 2001, Blagosklonny et al 2002, Park et al 2003, Goetz et al, 2001, Morimoto et al 1997, Young et al 2001)

Hsp90 client proteins play important roles in the regulation of the cell cycle, cell survival, cell growth, oncogenesis and apoptosis (Whitesell et al 1994). 17-AAG binds with a high affinity into the ATP binding pocket in Hsp90 and induces the degradation of proteins that require this chaperone for conformational maturation. 17-AAG is a less toxic analogue of Geldanamycin (GA), inducing apoptosis and displaying anti-tumour effects. The benzoquinone ansamycins, herbimycin A and GA, were first described as inhibitors of tyrosine kinases and were shown to reverse cell transformation by oncogenic kinases such as Src, Abl, and ErbB (Uehara et al, 1988). Later, it was shown that ansamycins do not affect kinases directly but instead serve as inhibitors of Hsp 90 (Whitesell et al, 1994), a chaperoning protein responsible for proper protein folding and an important participant in a variety of cellular processes (Richer et al, 2001) . GA acts by blocking the binding of ATP to Hsp 90 (Grenert et al, 1997), which leads to destabilization of Hsp 90 complexes with its client proteins rendering them available for proteasomal degradation (Whitesell et al, 1997, Schulte et al 1997). The ability of GA to deplete Raf1, ErbB2, and mutant p53 in breast cancer cells was found to correlate with its antiproliferative activity (Won et al 1997), making it a plausible candidate for use in cancer treatment. However, GA caused liver toxicity in preclinical studies (Supko et al, 1997). A search for more tolerable

derivatives has yielded 17-AAG, a less toxic analogue that retains the tumoricidal features of GA. Like its parent compound, 17-AAG inhibits several signaling pathways through binding to Hsp 90, which results in destabilization of signaling complexes and degradation of its client proteins by the proteasome in a variety of cell lines (Schulte et al, 1998, Kelland et al, 1999, Hostein et al, 2001). Treatment with 17-AAG has been shown to inhibit tumor growth and induce apoptosis in colon cancer, glioblastoma, and breast cancer cell lines (Hostein et al, 2001, Yang et al, 2001, Munster et al, 2001). These studies led to the active clinical development of 17-AAG as a potential anticancer drug (Wilson et al, 2001, Munster et al, 2001). Recent work suggests that 17-AAG enhances the cytotoxic effects of paclitaxel in non-small cell lung cancer cell lines and xenografts (Nguyen et al, 2001) and of Taxol and doxorubicin in breast cancer cell lines (Munster et al, 2001) , making it a promising candidate for combination treatment of solid tumors. These mechanisms may also be critical to an interaction with ansamycins because as has been previously shown, GA was able to inhibit signaling pathways using Hsp 90 and its analogues as chaperones.

In a Phase I trial at Memorial Sloan Kettering, the drug was administered daily for 5 days at 80mg/m^2 with peak plasma levels of 2700nM. The infusion was repeated every 3 weeks. The toxicities noted were diarrhoea, thrombocytopaenia, and transient transaminitis (Munster et al 2001). At the Royal Marsden Hospital in the UK, with weekly administrations at doses of 80mg/m^2 , no biochemical or haematological toxicities were observed. Further trials and in vitro analyses are required to observe the effects of this potential therapy on ATC.

1.10 Tamoxifen

Tamoxifen is the most successful chemotherapeutic agent in the history of medicine.

Traditionally, tamoxifen has been used as an oestrogen antagonist in the treatment of oestrogen receptor (ER) positive breast cancer as adjuvant therapy following surgical removal of tumour. The search for alternative mechanisms of action of tamoxifen was originally stimulated by the finding that the growth inhibition of human breast cancer cell lines in vitro with micromolar concentrations of tamoxifen could not be reversed by competition with large concentrations of oestradiol (Sutherland et al. 1983 & 1986), suggesting that tamoxifen may be acting at sites other than the oestrogen receptor.

The notion that tamoxifen influenced the cellular microenvironment in a breast tumour via the stromal compartment of the tumour was stimulated by the counter-intuitive clinical findings of the Nolvadex adjuvant tamoxifen trial (NATO 1987). The results of this trial were further confirmed by the Scottish MRC trial (1988) and the Early Breast Cancer Trialists Collaborative Group (1992). Taken together, these studies demonstrate that ER status does not predict a subgroup of patients that will respond to adjuvant tamoxifen therapy. There is increasing evidence that tamoxifen has many actions independent of its effect on the ER receptor.

Tamoxifen inhibits the action of protein kinase C (PKC), a calcium and phospholipid-dependent protein kinase, which forms a part of the second messenger system for many growth factors. Mutations in PKC have been shown to promote tumour growth (Guillem et al., 1987). Its action appears to be through inhibition of the phospholipid rather than directly on PKC (O'Brian et al., 1985).

Calmodulin is an intracellular calcium receptor involved in the cell cycle and its inhibition with the specific antagonist calmidazolium blocks progress of MCF-7 cells through the cell cycle (Musgrove et al. 1989). This action on cell proliferation was indistinguishable from pure oestrogen antagonists and nonsteoidal antioestrogens. Another key enzyme known to be inhibited by tamoxifen is Calmodulin-dependent cAMP phosphodiesterase (CDP) (Lam H-Y.P., 1984). Celeste et al (1990) found that tamoxifen may alter calcium-dependent processes by interacting directly with calmodulin.

Another interesting property of tamoxifen is that it appears to reverse multidrug resistance (Kirk JS et al. 1993a and b) and synergizes with vinblasine and adriamycin toxicity (Leonessa F et al. 1994), and with cisplatin cytotoxicity (McClay E et al. 1993) and delays the development of resistance to cisplatin in human melanoma and ovarian cell lines (McClay et al. 1994, Scambia g et al. 1992). This is felt to be mediated by its effect on glycolipid metabolism. Glycosphingolipids accumulate in multidrug resistant cancer cells. Tamoxifen acts on cellular lipid metabolism (Cabot MC et al. 1995, Kiss Z. 1994, Cabot MC et al. 1996) and on P-gp-dependent and independent modes (Kirk J et al. 1994, McClay E et al. 1993b), most likely affecting the participation of lipids on drug transport.

A number of studies have confirmed antiangiogenic mechanisms of tamoxifen. Tamoxifen treatment reduced blood vessel counts in MCF-7 mammary tumours in Swiss mice by approximately 70% (Linder and Borden, 1993). In a separate study, tamoxifen therapy reduced microvessel density of MCF-7 tumours implanted in mice by 50%, as

measured by magnetic resonance imaging and histochemical staining (Furman Haran et al, 1994). Tamoxifen inhibited angiogenesis in the chick egg chorioallantoic membrane assay (Gagliardi and Collins, 1993). As this effect was not reversed by the addition of excess oestrogen it suggests that antiangiogenic activity is by an ER-independent mechanism. One potential mechanism by which tamoxifen may reduce angiogenesis is by inhibiting the proliferation of endothelial cells and a number of studies have confirmed this. Tamoxifen reduced H³-thymidine uptake by HUVECs by 70%, an effect that was potentiated by the addition of shark cartilage (McGuire et al, 1994). Exposure of porcine pulmonary artery and human dermal microvascular endothelial cells to VEGF and bFGF, separately, increased the proliferation of these cells, but exposure to tamoxifen abolished this effect (Gagliardi et al, 1996). The effect was not reversed by an addition of excess 17-oestradiol, again suggesting that the effect of tamoxifen is independent of the ER receptor. Kimberley et al (2000) examined the effect of tamoxifen on a number of ER negative animal models; fibrosarcoma tumours, rat aortic rings and corneal pocket assays, and found that tamoxifen significantly reduced vessel formation, vascular sprouting and vascular length respectively in all cases.

Our own laboratory has confirmed that tamoxifen attenuates VEGF mediated angiogenesis in vivo (McNamara et al, 2000). As tamoxifen has proven effectiveness and safety in the clinical setting, exploration of its use as an anti-metastatic agent could be of benefit to patients with cancer types other than breast cancer. We investigated this response in ATC cells in vitro.

1.11 Specific Aims and objectives

Anaplastic Thyroid Cancer (ATC) remains the most aggressive cancer in nature with a dismal prognosis. Despite advances in treatment modalities, there are no internationally accepted regimen which improves survival. International audits quote this cancer at approximately 2-5 % of thyroid malignancies.

A. Epidemiology

1. Following acquisition of consent from twenty eight Irish consultants (whom the patients are tracked to, according to The National Cancer Registry, NCR) we will analyse the registry data to examine the epidemiology of ATC in Ireland between 1994 and 2004. Clinically relevant data regarding gender distribution, age, diagnosis, treatment and survival will be considered.

2. IHNOD. Irish Head and Neck Oncology Database. In an effort to evaluate our own patients, in cooperation and not in competition with the National Registry Database we will consider the DAHNO (Data for Head and Neck Oncology) database experience in England. We will establish a National database with all aspects of tumour features recorded. Using Microsoft Access we will develop a relational database using the variables listed. A relational database is a database that conforms to the relational model, and refers to a database's data and schema (the database's structure of how those data are arranged).

B. Oncology

3. The Her2/neu, ER-1, and EGF-R1 expression in anaplastic thyroid cancer will be investigated retrospectively using archival tissue from eight patients who had attend St Vincent's Hospital Dublin over a five year period from 1995 – 2005. The Her2/neu,

ER-1, and EGF-R1 expression will be immunohistochemically detected on sections from formalin-fixed, paraffin-embedded tissues using monoclonal antibody staining with Trilogy antigen retrieval and The Vector Elite Detection system to visualise the antibody-antigen complex. Our controls are ER positive human breast tissue, HER positive human breast tissue and human placenta for erb-1.

4. The anti-neoplastic properties of Tamoxifen will be investigated on Estrogen receptor negative CAL-62 and BHT101 Anaplastic Thyroid tumour cell lines. The anti-proliferative effects observed using colorimetric dimethyl-thiazol-diphenyltetrazolium bromide (MTT), pro-apoptotic effects observed using flow cytometry (annexin V). Tumour metastatic potential will be investigated with a Matrigel invasion assay with tamoxifen and chemotactic agents Vascular Endothelial Growth Factor and Epidermal Growth Factor.

5. We will investigate the anti-neoplastic properties of 17-Allylamino-17-demethoxygeldanamycin (17-AAG) on CAL-62 and BHT-101 Anaplastic thyroid cell lines. The anti-proliferative effects will be observed using colorimetric dimethyl-thiazol-diphenyltetrazolium bromide (MTT), pro-apoptotic effects observed using flow cytometry (annexin V). Tumour metastatic potential will be observed with a Matrigel invasion assay with 17-AAG and chemotactic agents Epidermal Growth Factor and Vascular Endothelial Growth Factor.

6. We will write ATC Review articles at the beginning and end of this thesis to assess the progression of oncology information in the literature over the 4 year period.

Chapter 2

Materials and Methods

2.2 Tissue Culture Techniques

An aseptic technique was used at all times for cell culture work. Cell culture was carried out in a grade II laminar flow cabinet (Holten Lamin Air HB2436, Allerod, Denmark). The cabinet was switched on at least 20 minutes prior to use and sterilised by swabbing with 70% ethanol in water before and after use. All equipment and reagents brought into the cabinet were sanitized in a similar manner before being brought into the cabinet. Disposable gloves and a clean lab coat were worn at all times.

2.2.1 Cell Typia

CAL-62 and BHT 101 cell lines were obtained as frozen with 2×10^6 cells/ampoule from The German cell library (DSMZ). These cells were cultured in 75mm² flasks (Falcon, Lincoln Park,NJ) in complete medium 199 supplemented with 20% fetal calf serum, 5 mls of penicillin (100U/ml) and streptomycin sulphate (100µg/ml) solution, heparin (16U/ml), (Gibco-lifesciences). Cells were grown at 37°C in humidified 5% CO₂ conditions for 24 hours, until confluent monolayers were reached. The cells were then subcultured by trypsinization with 0.05% trypsin. 75% confluent cell cultures in the growth phase were used for all experiments.

The CAL-62 cell line was established from the thyroid gland (right lobe) of a 70-year-old woman with thyroid anaplastic carcinoma in 1988 and is described as being epithelial-like cells growing in monolayers which are tumourigenic in heterotransplanted nude mice. The BHT 101 cell line is a human thyroid carcinoma established from the lymph node metastasis of a 63-year-old woman with anaplastic papillary thyroid carcinoma; cells were described to not produce hormones, but to be partly positive for

thyroglobulin- and thyroxine (T4) (Palyi et al. 1993), epithelial-like cells growing in monolayers

2.2.2 Cell counting and viability

20 µl of the cell suspension to be counted was mixed with 180 µl of Trypan blue vital dye (Sigma, St Louis, Mo, USA) to yield a 1:10 dilution. Cells were counted using a Neubauer haemocytometer (Sigma Sigma, St Louis, Mo, USA). This chamber is essentially a microscope slide with a counting grid etched into the glass. The volume of this grid when the cover slip was carefully slid onto the slide (until Newton rings were noted) is precisely 0.0025mm². Cells are added to the slide by capillary action. The chamber was examined by phase-contrast microscopy using a x 40 objective (Nikon TMS, Tokyo, Japan). Cell counts were made inside a 16-squared area of the chamber excluding cells resting on the outside lines. Cells that did not exclude the trypan blue and stained blue were considered dead and counted separately. Counts of four sets of 16 squares were performed and the mean of these determinations was calculated. The mean number of cells per ml of fluid was then determined as follows:

$$\text{cell density} = \text{mean number of cells counted per field of 16} \times 10(\text{dilution factor}) \times 10^4$$

$$\% \text{ Viability} = \frac{\text{number of live cells counted}}{\text{Total number of cell counted}} \times 100$$

2.3 Flow cytometry

Cells were acquired on a FACScan (Becton Dickinson, Oxford UK) flow cytometer equipped with an argon laser excitation wavelength of $\lambda_{\text{ex}} = 488 \text{ nm}$. Fluorescence signals at $\lambda_{\text{em}} = 520 \text{ nm}$ (green fluorescence) and 580 nm (orange fluorescence) together with forward light scatter (FLS) and side light scatter (SLS) were recorded. 10,000 events were collected per sample, the data was registered on a logarithmic scale and analysed on CellQuest® software (Becton Dickinson, Oxford UK). The instrument was calibrated daily with Calibright beads (Becton Dickinson, Oxford, UK).

2.3.1 Apoptosis

The annexin V/ propidium iodide dual staining method of apoptosis was selected for analysis to allow simultaneous detection and differentiation between apoptotic and necrotic cell death. Therefore apoptosis was assessed using the TACS ® Apoptosis detection kit (R&D Systems Minneapolis, USA) according to the manufacturer's instructions. 2×10^5 cells were pelleted in microfuge tubes (Starstedt) by centrifugation at $300 \times g$ for 5 min. The tubes were inverted and blotted dry to remove all remaining medium, which is a critical step for annexin v binding. 100 μl of reaction mix (79 μl H_2O , 1 μl Annexin V, 10 μl propidium iodide, 10 μl 10x buffer) was added per sample and the samples were vortexed. The samples were incubated for 15 min at room temperature in the dark. 500 μl of 1x buffer was added and samples were analysed immediately by flow cytometry. Apoptosis was assessed at 6 and 24 h.

Two control samples were prepared to facilitate calibration of the instrument settings for the FACScan set up. An Anaplastic sample stained with annexin only (89 μ l H₂O, 1 μ l Annexin V, 10 μ l 10x buffer) was used to set the FL2 detector background. An Anaplastic sample stained with PI only (80 μ l H₂O, 10 μ l 10x buffer) was used to set the FL1 detector background. Membrane staining with FITC-labelled annexin V, indicates exposure of phosphatidylserine at the outer layer of the cytoplasmic membrane, a marker of early stage apoptosis. Dual staining with propidium iodide facilitates the detection of necrotic cells or cells with compromised membranes which would allow entry of annexin V to the inner leaflet of the membrane. Apoptotic cells stain positive only for annexin V where as necrotic cells stain positive for both annexin v and PI. (fig 2.1) .

2.4 Cell Morphology

May-Grunwald Giemsa staining was used to assess Anaplastic morphological changes and further confirm apoptosis in a selection of the Anaplastic samples. Anaplastic cell cytospin preparations were made using a Shandon Cytospin® (ThermoShandon, Cheshire, UK.) set at 10 x g for 3 min after 6 and 24 h of incubation as detailed in experimental methods and differentially stained using a commercial May-Grunwald Giemsa stain (Diff-Quick, Baxter Healthcare Products, Co Mayo, Ireland). Cells were assessed for apoptotic morphology microscopically (Pongracz et al, 1999). Cell shrinkage, nuclear condensation and increased vacuole formation are the morphological characteristics of apoptotic cell death.

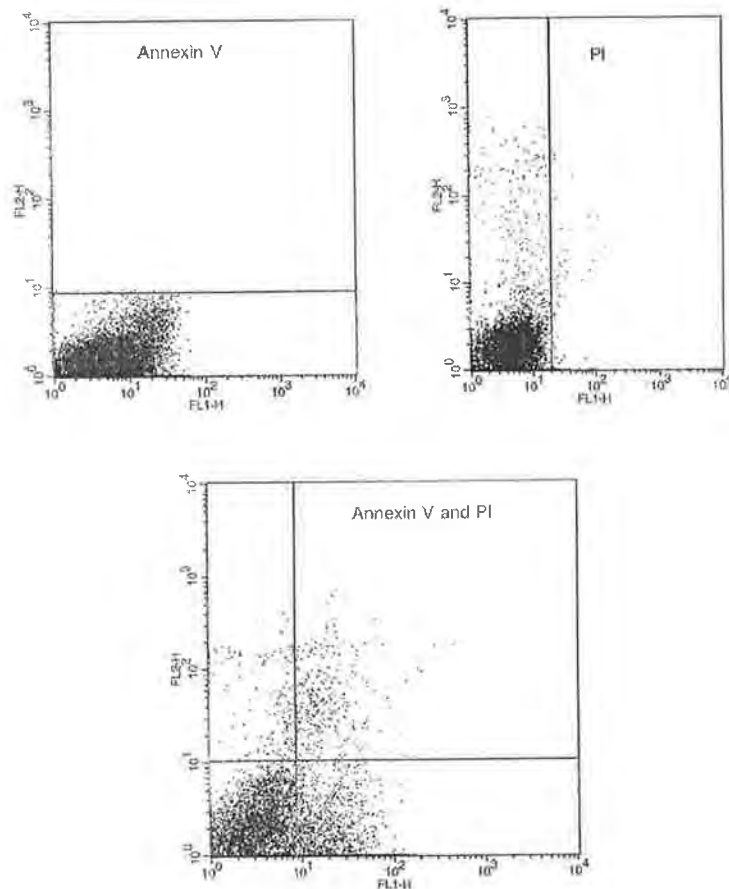


Fig 2.1

Control samples stained with annexin V only or propidium iodide only were used to calibrate the instrument for FACSscan set up and to place the quadrant markers on the dot plot. These quadrants are used to calculate the percentage apoptosis in dual-labelled samples where the lower right quadrant, cells positively stained for annexin V only, represents apoptotic cells and the upper right quadrant, positive for both annexin V and propidium iodide, represents necrotic cells. The lower left quadrant is unstained cells, which are alive. The upper left quadrant, cells stained only with propidium iodide, acts as an internal control for the experiment as this artifactual staining happens if cell membranes are disrupted as a result of the experimental conditions. This population should be less than 5%.

2.5 Preparation of Cell Lysates.

After decanting cell culture medium from flasks, cells were washed three times in cold 1' PBS. In experiments examining proteins implicated in apoptosis, cell culture supernatant was collected and centrifuged at 300 g for 5 min to pellet any floating (apoptotic) cells. These were then combined with adherent cells in the appropriate flasks. Ice cold lysis buffer [5 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton-X 100, 0.5% (w/v) SDS (sodium dodecyl sulfate), 0.5% (w/v) deoxycholic acid, 1 mM PMSF (phenylmethylsulfonyl fluoride)] was added to each flask (300 µl per 25 cm² flask, 1 ml per 75 cm² flask) and left on ice for 1 h. Lysed cells were collected using cell scrapers and cell lysates were passed through a 1 ml U-100 insulin syringe (Braun Petzold, Germany) 10 times to shear genomic DNA and boiled for 10 min at 95°C on a heating block (Techne, Cambridge, UK) before storing at -80°C.

2.6 Determination of Protein Concentration.

Protein concentrations were determined using the BCA (Bicinchoninic acid assay) reagent kit (Pierce, IL, USA). Based on the Biuret reaction, where Cu²⁺ is reduced to Cu⁺ by protein in an alkaline medium, the assay is highly sensitive and allows the colorimetric detection of the copper cation Cu⁺, using a specified reagent containing bicinchoninic acid. Cell lysates were diluted 1:10 in PBS prior to analysis. 10 µl of each protein standard (0 mg/ml-1000 mg/ml) and protein lysates were added in duplicate to a 96-well plate. A working reagent consisting of an alkaline bicarbonate solution (BCA Reagent A) mixed in a 50:1 ratio with a copper sulphate solution (BCA Reagent B) was prepared. 200 µl was added to each well containing protein standards and cell lysates and incubated at 37°C for 30 min. Absorbance values were read at 570 nm using the

Multiskan Ex microplate reader (Labsystems, Helsinki, Finland). Protein concentrations were calculated from a standard curve.

2.7 Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE).

A 12% (v/v) separating gel was prepared as follows; 9.2 ml 30% (w/v) acrylamide:bisacrylamide (29:1), 4.5 ml Tris-HCl pH 8.8, 8.3 ml distilled water, 176 μ l 10% (w/v) SDS, 8 μ l TEMED, 120 μ l 10% (w/v) APS, which was prepared fresh each day. The solution was mixed and gels were cast in upright glass plates (Atto Corporation, Tokyo, Japan). Ethanol (70%) was layered on top of the gel mix to aid polymerisation and to remove any air bubbles. When set, the ethanol was gently removed and the top of the gel was rinsed with distilled water. A 5% (v/v) stacking gel containing 1.7 ml acrylamide, 2.0 ml Tris-HCl pH 6.8, 6.0 ml distilled water, 100 μ l 10% (w/v) SDS, 10 μ l TEMED and 150 μ l 10% (w/v) APS was prepared. Once added to the top of the separating gel, a 12-well comb was inserted and the gel was allowed to set.

2.8 Protein Electrophoresis and transfer.

Equal concentrations of protein lysates were prepared in 2 x laemmli buffer (2 ml Tris HCl pH 6.8, 5 ml 10% (w/v) SDS, 1 ml 2-Mercaptoethanol, 2 ml glycerol, 0.05 g (w/v) bromophenol blue) and denatured at 95°C for 10 min. Samples, including protein marker (New England Biolabs Ltd., UK), were separated at 25 mA per gel in electrode buffer (50 mM Trizma Base, 384 mM Glycine, 0.1% (w/v) SDS) until such time as the dye front reached the bottom of the gel. Proteins were subsequently transferred to a nitrocellulose membrane (PALL Corporation, FL, USA) using a Trans-blot transfer apparatus (BioRad

Laboratories, CA, USA). The gel was placed against the nitrocellulose membrane in a sandwich of Whatman paper (Whatman Laboratory Division, Maidstone, UK) and protected on either side by sponges. The assembly was placed in the BioRad transfer apparatus. Proteins were transferred in 1x transfer buffer (0.15 M glycine, 20 mM Tris, 0.1% (w/v) SDS, 20% (v/v) methanol) overnight at 4°C at 90 mA using a cooling apparatus (Medingen, Bonn, Germany).

2.9 Western Immunoblotting.

Following the transfer of proteins, membranes were blocked in 5% non-fat dry milk (Marvel) in Tris-buffered saline (25 mM Tris-HCl pH 7.6, 150 mM NaCl) containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Primary antibody was diluted in 5% non-fat dry milk in TBST according to that specified by the manufacturer and incubated on a shaker table for 1-2 h. Membranes were washed 6 times for 5 min each in TBST. A 1:2000 dilution of the appropriate species-specific HRP conjugate (Dako, Glostrup, Denmark) was then added for a further 1.5 h. Membranes were washed six times for 5 min each in TBST. Bound antibody complexes were detected using the Supersignal West Pico Chemiluminescent substrate kit (Pierce, IL, USA).

2.10 Immunohistochemical Staining

2.10.1 Specimen processing

Four µm sections were cut and mounted on vectabond-coated slides (Vector Laboratories, USA) and dried overnight at 55°C. The sections were deparaffinised (including antigen retrieval) using Trilogy (Cell Marque) which combines the three pre-treatment steps used

in immunohistochemistry: deparaffinisation, rehydration and unmasking of antigenic sites. The working solution was prepared as a 1:20 dilution with distilled water. 24 slides per slide rack were dewaxed in 200 ml of diluted Trilogy solution by heating for 15mins in a pressure cooker (Kenwood) on low. Slides were immediately transferred from the first Trilogy treatment slowly into a fresh Trilogy staining dish for a hot rinse, agitated and let stand for 5 minutes. The Trilogy was washed off with distilled water for 5 minutes. Slides were immersed in 0.3% hydrogen peroxide blocking solution (2.0 ml of 30% v/v hydrogen peroxide solution (BDH 101284N) to 200ml of distilled water) for 10 minutes to inhibit endogenous peroxidase activity. The sections were placed in deionised water for 5 minutes

2.10.2 Immunohistochemistry Reagents

Normal Horse Serum (NHS)

Vector Laboratories S2000. Prepared as a 1:10 dilution made up with TBST.

Oestrogen Receptor

Rabbit monoclonal antibody (clone SP1) supplied by LabVision (RM-9101-S). Prepared as a 1:50 dilution with TBST. The antibody is incubated for 1 hour at room temperature.

VEGF antibody

Polyclonal antibody supplied by Santa Cruz Research Products. Prepared as a 1:100 dilution with TBST. The antibody is incubated for 30 minutes at room temperature.

Vector Elite Detection System

The secondary antibody solution is prepared by adding 20µl of NHS (Normal Horse Serum) and 20µl of Universal Biotinylated antibody to 1000µl of TBST. The working

avidin-biotin complex is prepared by adding 20 μ l of Reagent A and 20 μ l of Reagent B to 1000 μ l of TBST.

2.10.3 Reagent preparation

0.1M Tris Buffer pH 7.6

Tris buffer is used to prepare Tris buffered saline and as a DAB diluent (Diaminobenzidine) and is made up fresh before an immunohistochemical run. 12.11g of DAB diluent was allowed to dissolve in 500ml of dH₂O + 80ml of 1M HCL on the magnetic stirrer. When the Tris had dissolved the pH of the solution was checked and adjusted to pH7.6 with 1M HCl or 1M NaOH. The final volume was made up to 1 l with distilled water.

0.05M Tris Buffered Saline/Tween (TBST) pH 7.6

Tris buffered saline is used as a wash buffer and antibody diluent in immunohistochemical procedures and is made up fresh at the start of each week. 8.77 g of Sodium Chloride (NaCl) was added to 800ml of Tris container and add the required volume of Tris buffer on the magnetic stirrer. When the NaCl had dissolved the pH of the solution was adjust to pH7.6 with 1M HCl if necessary. 500 μ l of Tween 20 was added and made up to final volume of 100ml with distilled water. The final solution contains 0.05M Tris and 0.15M NaCl.

DAB stock and working solutions

A DAB 100mg vial (Sigma D9015) was removed from the freezer 10 minutes before preparation. 20ml fresh 0.1M tris buffer pH7.6 was added slowly to the vial using a 20ml syringe and new needle. The DAB solution was aliquotted into 1ml amounts and stored the aliquots at -20°C until required. Each aliquot contained 5mg (0.5%) of DAB. To

prepare the DAB working solution a DAB stock solution aliquot was defrosted 30 min before use taking care to avoid oxidation due to light exposure and 9mls of 0.1M tris buffer pH7.6 was added and immediately prior to use 10 (l of H₂O₂ was added to the DAB solution. DAB is a potential carcinogen and must be disposed of in a safe manner by inactivation with sodium hypochlorite

2.10.4 Immunohistochemistry

The expression of Oestrogen Receptors (OR), Epidermal Growth Factor and Vascular Endothelial Growth Factor was investigated with immunohistochemical staining using monoclonal antibodies. After rinsing with Tris buffered saline and incubating with normal horse serum for 10 minutes, the sections were incubated with the primary antibodies OR and VEGF for 30 minutes, followed by three washes with TBS.

The Vectastain(ABC system (Vector Laboratories - PK6200) is used to perform routine immunohistochemical staining and is based on the avidin/biotin binding system. Once the primary antibody has bound to its specific antigen the universal secondary antibody then binds to primary antibodies (will detect antibodies raised in mouse or rabbit). The secondary antibody is biotinylated and once the avidin/biotin complex is added, the biotin molecule on the secondary antibody binds to the free biotin site on the avidin/biotin complex. Peroxidase molecules are conjugated to the ABC complex and these then react with 3,3-diaminobenzidine tetrahydrochloride (DAB - Sigma D9015) in the presence of hydrogen peroxide to produce a water-insoluble brown end product at the site of the antigen-antibody. This system was used to visualise the antibody-antigen complex. Briefly, the sections were treated with biotinylated horse immunoglobulin for 20 minutes,

followed by three washes with TBS. The slides were then treated with peroxidase-conjugated avidin-biotin complex for 20 minutes. After further washing in TBS, the slides were developed with 0.01% H₂O₂ in 0.05% DAB for 5 minutes, followed by a wash with deionised water.

2.10.5 Haematoxylin Counterstain

The slides were immersed in deionised water for 5 minutes and lightly counterstained with Harris' haematoxylin by dipping the slides 10 times into haematoxylin bath (Ortho Modification II - CellPath RBA4213-00A). The slides were then "blued" in warm running water for 1-3 min and then dipped twice into an acid alcohol bath. The nuclear staining was checked microscopically to ensure appropriate staining. If there was excessive Haematoxylin staining the slides were dipped in 1% acid alcohol for ~3 sec. Slides were again rinsed under running water for 1 min. Finally the slides were dehydrated by dipping in 70% ethanol and in 100% ethanol 10 times. The slides were mounted with DPX mounting medium (Sigma, Dorset, UK) and coverslipped.

2.10.6 Herceptin Assay

The HercepTest(is a semi-quantitative immunohistochemical assay used to determine Her-2 protein expression in formailin-fixed paraffin-embedded tissues.

Slides were warmed for 10 minutes in a 55(C oven before re-hydration through the five ultraclear troughs and all of the alcohols in the Medite Cot-20 staining machine for 5 minutes. Slides were rinsed in distilled water for 5 minutes and carefully checked to ensure successful dewaxing and rehydration. Slides were then immersed in the epitope

retrieval solution for 40 min and then allowed to cool in the solution for 20 min at room temperature (RT). Slides were rinsed with fresh HercepTest(wash buffer followed by 5 min incubation in fresh HercepTest(wash buffer. The slides were drained of excess buffer and wiped around the tissue section using a lintless tissue. Enough HercepTest(peroxidase blocking reagent was applied to cover the sections and incubated for 5 min. Slides were gently rinsed twice with wash buffer for 3 minutes each. The slides were drained of excess buffer and wiped around the tissue section using a lintless tissue and 100(1 of primary antibody was added to the test sections with positive control and 100(1 of negative control reagent to the negative control sections. The slides were incubated for 30 min. Slides were rinsed with fresh HercepTest(wash buffer followed by 5 min incubation in fresh HercepTest(wash buffer. The slides were drained of excess buffer and wiped around the tissue section using a lintless tissue and 100(1 of visualisation reagent was added to all sections and incubated at RT for 30 min. Slides were rinsed well in distilled water. One drop (approximately 25 (l) of DAB chromogen added to 1ml of DAB buffered substrate and applied to the tissue sections for 10 minutes. Slides were rinsed well in distilled water and counterstained with haematoxylin (2.10.4).

The HercepTest™ kit and scoring method from Dako-Cytomation uses a scale of 0, 1+, 2+ and 3+ to score the expression of HER-2/neu protein by measurement of staining intensity, staining localisation, pattern continuity and tissue homogeneity. HercepTest(is interpreted as negative for HER2 protein overexpression if there is no staining at all, or membrane staining in less than 10% of the tumour cells (0 score). A faint/barely perceptible membrane staining detected in more than 10% of the tumour cells is scored as 1+, these cells are also only stained in part of the membrane. A weak to

moderate staining of the entire membrane observed in more than 10% of the tumour cells is scored as 2+ and a strong staining of the entire membrane observed in more than 10% of the tumour cells is scored as 3+.

A supplied performance control slide, containing three pelleted, formalin-fixed, paraffin-embedded cell lines was included in each staining procedure and used to validate the staining procedure.

- Negative cell line (MDA-231) – No membrane staining observed.
- 1+ cell line (MDA-175) – Faintly perceptible staining intensity with incomplete membrane staining present on small to moderate numbers of the cells.
- 3+ cell line (SK-BR-3) – Intense, complete membrane staining present on the vast majority of cells.

Score: 0 Her-2 Overexpression Assessment: Negative

No staining is observed or membrane staining is observed in less than 10% of the tumour cells



Score: 1+ Her-2 Overexpression Assessment: Negative

A faint/barely perceptible membrane staining is detected in more than 10% of the tumour cells. The cells are only stained in part of



their membrane.

Score: 2+ Her-2 Overexpression Assessment: Weak Positive

A weak to moderate complete membrane staining is observed in more than 10% of the tumour cells.



Score: 3+ Her-2 Overexpression Assessment: Strong Positive

A strong complete membrane staining is observed in more than 10% of the tumour cells.



2.11 Matrigel ‘In Vivo Replication’ Invasive Model

The Boyden chamber assay, originally introduced by Boyden for the analysis of leukocyte chemotaxis, is based on a chamber of two medium-filled compartments separated by a microporous membrane. In general, cells are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents are present.

The originally described Boyden Chamber method was modified by using a transwell polycarbonate membrane (Costar, Cambridge, MA). Matrigel diluted in 4H medium was solidified onto a transwell polycarbonate membrane. CAL - 62 and BHT - 101 cells 2×10^5 cells/well suspended in Media with a combination of Fetal Calf serum added at 10% CAL - 62 and 20% for the BHT - 101 and Tamoxifen or 17 AAG at varying concentrations.

Wells were incubated at 37C in humidified air containing 5% CO₂ for 24 hrs. The cells on the upper surface of the polycarbonate membrane were wiped off, and the remaining cells that transversed the Matrigel and spread on the lower surface of the filter were collected separately. The filter was stained using a 'Gurr Rapid staining set for microscopy' (BDH). Hema Gurr is a staining set for rapid manual staining. It consists of a fixing solution, two buffered staining solutions and buffer tablets.

The staining pattern of the filter corresponds to the classical staining patterns, cell elements and structures being stained distinctly. Morphological alterations are thus easily recognized. The air-dried blood smear is fixed and stained by immersion into each solution sequentially. The staining time is approximately 30 seconds.

1. Transfer each solution into a separate jar with a lid or into a staining trough.
2. Prepare filter and dry in air
3. Immerse filter five times for one second each time into fixing solution. Allow the solution to drip off.
4. Immerse the filter three times for one second each time into staining reagent 1.
5. Immerse the filter six times for one second each time into staining reagent 2. Allow the solution to drip off.
6. Rinse with buffer solution pH 7.2. Prepare the buffer solution by dissolving 1 buffer tablet in 1 litre of freshly distilled water. The buffered distilled water is stable for at least 4 weeks when stored in a tightly closed glass bottle.
7. Dry and mount.

The staining solutions should be discarded when there are deviations from the usual colour patterns. When the fixing solution has been used up, methanol can also be used for fixing.

Counting of the cell can be achieved by a computer or by eye. The entire filter is surveyed as the distribution of the cells may be uneven. The most representative sample is taken, counting with a 10x magnification using a grind has be proven to be reliable and reproducible. This was our chosen method (Liotta et al, 1989).

MTT Proliferation Assay

MTT Cell Proliferation Assay is an experiment for the determination of cell number using standard microplate absorbance readers. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by metabolic active cells. The formazan is then solubilized, the concentration then determined by optical density at 570 nm. The result is a colorimetric signal proportional sensitive assay to the cell number

Method

1. Plate cells into 96-well tissue culture plates. Cells are seeded at densities between 5000 and 10,000 cells per well since they will reach optimal population densities within 48 to 72 hours.
2. Experiments are carried out by adding the chemicals agent (s) into the appropriate well. The volume of tissue culture medium in each well should be 0.1mL, and the medium may contain up to 10% Fetal Bovine Serum.
3. One vial of MTT solution should be used for each 96-well plate assay.

Note: If sediment is present in the solution, heat the solution to 37°C and swirl gently until a clear solution is obtained.

4. Add 10µL MTT solution to each well. Mix by tapping gently on the side of the tray or shake briefly on an orbital shaker.
5. Incubate at 37°C for 4 hours. At high cell densities (>100,000 cells per well) the incubation time can be shortened to 2 hours.
6. Remove medium and add 200µL DMSO (Dimethyl sulfoxide) into each well to dissolve the formazan by pipetting up and down several times.
7. Measure the absorbance on an ELISA plate reader with a test wavelength of 570 nm.

2.11 Epidemiology Methodology

At a national level there is a responsibility to ensure that clinical audit is an integral part of the quality improvement for Governance strategies and Clinical Guidelines. Clinical guidelines aim to improve quality of healthcare. They can improve outcomes by providing recommendations via audit analysis of existing databases for treatment in the many aspects of a Head and Neck cancer management.

We approached 'The National Cancer Registry' data to examine the epidemiology of ATC cancer in The Republic of Ireland between 1994 and 2004 searching the known information for clinically relevant data about stage, treatment and survival. The Registry was fully supportive and encouraged further appraisal of their information. Indeed they highlighted to us the difficulties that exist in accumulating high quality oncology data.

Northern Ireland's database were also examined from 1993 to 2003. No further patient file investigation took place from the Northern Irish patient population. Basic medical record numbers and tracking Physicians were supplied.

We then identified the Physicians and Surgeons each of these cases were tracked to according to the Cancer Registry. We then verified the findings held by the Cancer Registry and conducted a review of all the information known from each case. We examined a number of variables from the basic information supplied by The Registry. Detailed patient file investigation then took place.

We established a comprehensive database using Microsoft Excel to conduct a nationwide audit. In particular the fields of enquiry were the histopathological diagnosis, sex, gender, metastatic deposit sites, effects of smoking, survival time and treatment strategies employed.

This research was conducted in full cooperation with The Cancer Registry and the consent of every Physician/ Surgeon contacted. The results were presented at the Irish Otolaryngology Society (IOS) meeting in 2007.

2.12 Statistics

Data are expressed as mean \pm standard error of the mean (SEM). Normally probability plots were examined to determine if the data was normally distributed. For normally distributed data, statistical difference between two groups was determined using the t-test, where a P value of <0.05 was taken as a significant. Analysis of Variance (ANOVA) was used for 3 or more groups in an experimental comparison with a LSD

post Hoc correction. Fisher's exact test was used for incident data. Statistical analysis performed using SPSS ® (version 11) software.

Chapter Three

The Epidemiology of Anaplastic Thyroid Cancer International Profile & Irish National Audit Results.

3.1 The Global and Irish Experience:

3.1.1 National Audit Results 1994-2004

We began this thesis by conducting an audit to estimate the impact of this disease nationally. We contacted The National Cancer Registry in Cork and The National Cancer Registry in Northern Ireland through Queens University, Belfast to have a complete Irish Perspective.

In accordance with the recent guidelines as stated by The National Institute of Clinical Excellence we defined our purpose and began the audit process. (Principles for Best Practise in Clinical Audit, National Institute for Clinical Excellence 2004). Our aim was to establish the number of cases of this disease in all its histologic forms. We sought consent from all of the Doctors these patients were traced to within the country. In total we contacted 21 consultants (to whom the registry traced their patients) and received written consent to evaluate each of their cases. All of the information was then correlated and statistically analysed.

We found that in Ireland there are higher levels of this disease in comparison to international statistics and furthermore our figures may indeed be very conservative. No current treatment strategy in Ireland offers improved survival advantage. Histopathological differentiation of Anaplastic thyroid cancer carries equal prognosis. Smoking encourages earlier presentation with our smokers presenting over one decade earlier. Our selection criteria were in accordance with the ICD – Oncology characterisation of disease and IARC International Agency for Research in Cancer. This is the World Health Organisation format. The raw data was analysed and presented for discussion at the National Otolaryngology Meeting in October 2006.

A total of 812 cases of Thyroid Cancer were recorded in The Republic of Ireland with 51 cases of Anaplastic Thyroid Cancer. This represents 6.28% of diagnosed thyroid cancers. Northern Ireland had 515 cases with 25 cases of Anaplastic Thyroid Cancer which represents 4.85%.

Further analysis revealed a very wide age range (55-93 years) with a predominance of females and survival strongly influenced by gender, age and histological type. There was limited data about the surgical procedures undertaken. The database listed those who received Chemotherapy or Radiotherapy however doses and regimen were not listed.

Cancer registry data is well able to sustain an analysis of the epidemiology of thyroid cancer but further work is necessary to improve the quality of clinically relevant information about stage and treatment that could be used for audit

The objective of this study was to define the epidemiology of thyroid cancer in our regional population and compare results with Surveillance, Epidemiology, and End Results (SEER) Program cancer registry trends.

3.2 Surveillance, Epidemiology, and End Results (SEER) (Davies et al 2006)

While the incidence of some other cancers has declined over time, the incidence of thyroid cancer has increased greatly in recent years. There are at least two possible explanations for this trend: it is possible that thyroid cancer is truly occurring more frequently than in the past, but it is also possible that it is simply being detected more often. Techniques such as ultrasound and fine-needle biopsy, for example, may allow doctors to identify thyroid cancer that would otherwise have gone undetected.

Detection of a cancer that would never otherwise have caused health problems is referred to as overdiagnosis. As a result of overdiagnosis, some individuals receive cancer diagnoses and treatments that they might otherwise have avoided. To explore the reasons for the recent increase in thyroid cancer incidence, we evaluated a respective cohort of patients using the Surveillance, Epidemiology and End Results programme and data on Thyroid cancer mortality from the National Vital Statistics System. Between 1973 and 2002, the incidence of thyroid cancer increased by 2.4 fold (from 3.6 cases of thyroid cancer per 100,000 people per year to 8.7 cases of thyroid cancer per 100,000 people per year). Most of the increase was due to an increase in small papillary thyroid cancers. The number of deaths from thyroid cancer remained stable during this time period with approximately 0.5 deaths per 100,000. There was a significant change in the incidence of papillary thyroid cancer which increased from 2.7 to 7.7 per 100,000, a 2.9 fold increase. Table 3.1 reports thyroid cancer incidence and mortality in Europe, and the World in 1998. We have a worrying Irish trend of high incidence, and, high mortality levels compared to World values. Figure 3.2 is a graph which observes the age adjusted incidence rates of thyroid cancer from the SEER database. It is interesting to note the discordance between the sexes. The increase in thyroid cancer incidence was due to an increase in the number of small tumors with 87% of the increase due to papillary cancers. There was no increase in the death rate.

Increasing cancer incidence is typically interpreted as an increase in the true occurrence of disease but may also reflect changing pathological criteria or increased diagnostic scrutiny. Changes in the diagnostic approach to thyroid nodules may have resulted in an increase in the apparent incidence of thyroid cancer. This is complicated by deciding who

requires investigation and who doesn't. Twenty percent of American adults have a palpable thyroid gland and 67% have a mass that can be visualised on ultrasound. (Ezzat et al, 1994)

These statistics, combined with the known existence of a substantial reservoir of subclinical cancer and stable overall mortality, suggest that increasing incidence reflects increased detection of subclinical disease, not an increase in the true occurrence of thyroid cancer. Increased detection of very small thyroid cancers may lead to unnecessary treatment in some patients and conjures significant ethical debate.

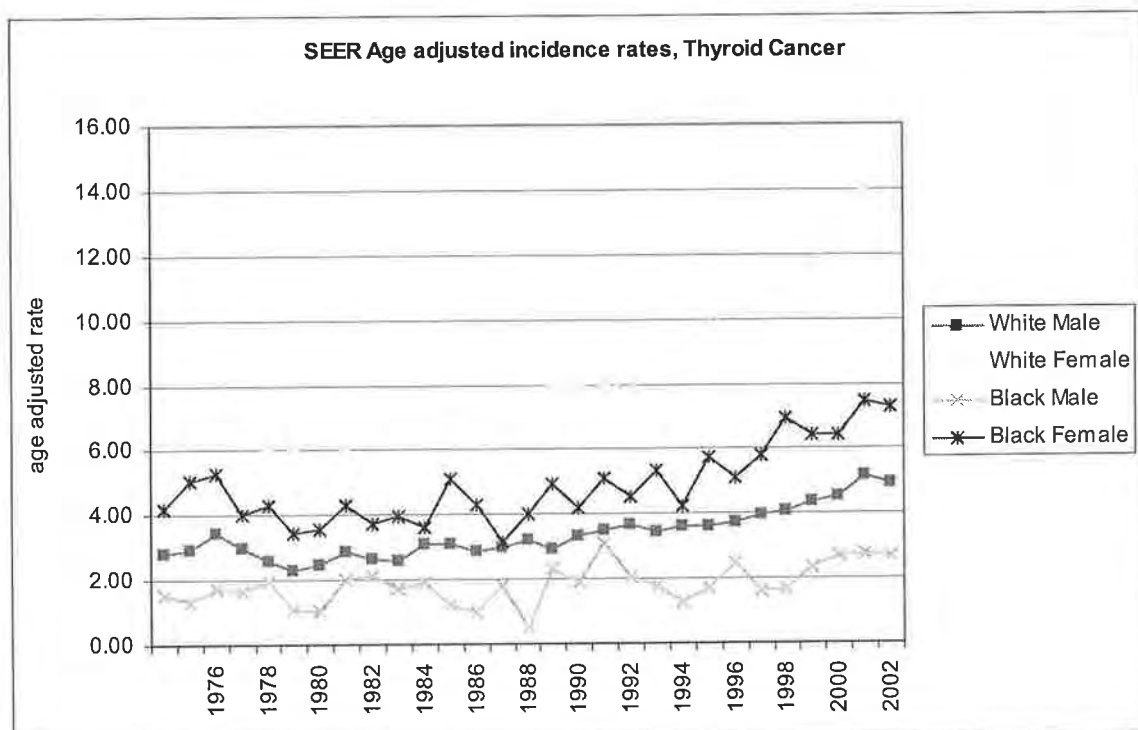


Fig 3.2 SEER incidence rates of Thyroid Cancer in the USA.
SEER Database (Davies et al 2006).

What is also alarming is the apparent increase in the numbers of women presenting with thyroid cancer. The American Cancer Society estimates 37,340 new cases of Thyroid cancer in 2008 (American Cancer Society 2008). They confirm it is much more common

in women. Of the new cases, about 22,590 will occur in women, and 7,590 in men however overall this is one of the least deadly cancers. The 5-year survival for all cases is nearly 97%. An estimated 870 women and 630 men (1,500 total) will die of thyroid cancer during the year 2006. What is of great concern is that of those who die, over 50% are due to the anaplastic variant.

	INCIDENCE				MORTALITY			
	Females		Males		Females		Males	
	EASR	WASR	EASR	WASR	EASR	WASR	EASR	WASR
European Union	2.27	1.85	5.58	4.53	0.53	0.36	0.68	0.44
Austria	3.22	2.72	6.12	4.99	1	0.64	1.2	0.77
Belgium	1.53	1.29	3.59	2.91	0.56	0.37	0.54	0.35
Denmark	1.52	1.21	3.15	2.66	0.52	0.36	0.69	0.44
Finland	3.02	2.36	9.21	7.49	0.74	0.49	0.83	0.51
France	3.8	3.06	7.41	5.94	0.5	0.33	0.61	0.39
Germany	2.2	1.79	4.57	3.57	0.7	0.46	0.87	0.55
Greece	1.3	1.07	3.4	2.8	0.35	0.24	0.46	0.29
Ireland	0.56	0.34	1.59	1.08	0.25	0.15	0.58	0.37
Italy	3.26	2.68	9.44	7.99	0.64	0.42	0.78	0.51
Luxembourg	0.8	0.68	5.58	4.35	0.42	0.32	0.93	0.61
The Netherlands	1.12	0.87	2.78	2.26	0.25	0.16	0.55	0.35
Portugal	1.6	1.28	6.61	5.29	0.36	0.23	0.78	0.51
Spain	2.12	1.75	5.42	4.31	0.46	0.32	0.58	0.37
Sweden	1.72	1.35	3.81	3.08	0.54	0.36	0.51	0.31
United Kingdom	0.91	0.74	3.03	2.52	0.36	0.24	0.46	0.29

Table 3.1 Thyroid Cancer Incidence and mortality in Europe, (EUCAN: 1998)

Thyroid Cancer Incidence and mortality in Europe, 1998

The International Agency for Research on Cancer (IARC), which is part of the World Health Organization (WHO), estimates 24,800 new diagnoses of thyroid cancer in the European Countries in 2000, (IARC, 2000). Although this cancer often has a good prognosis, approximately 3,890 European women and 2,103 European men died from thyroid cancer in the year 2000. The incidence appears reduced in Ireland compared to the European and world figures. (Table 3.1). Worldwide increased incidences has been associated with improve pick of early cancer by ultrasonography.

3.3 Irish Statistics

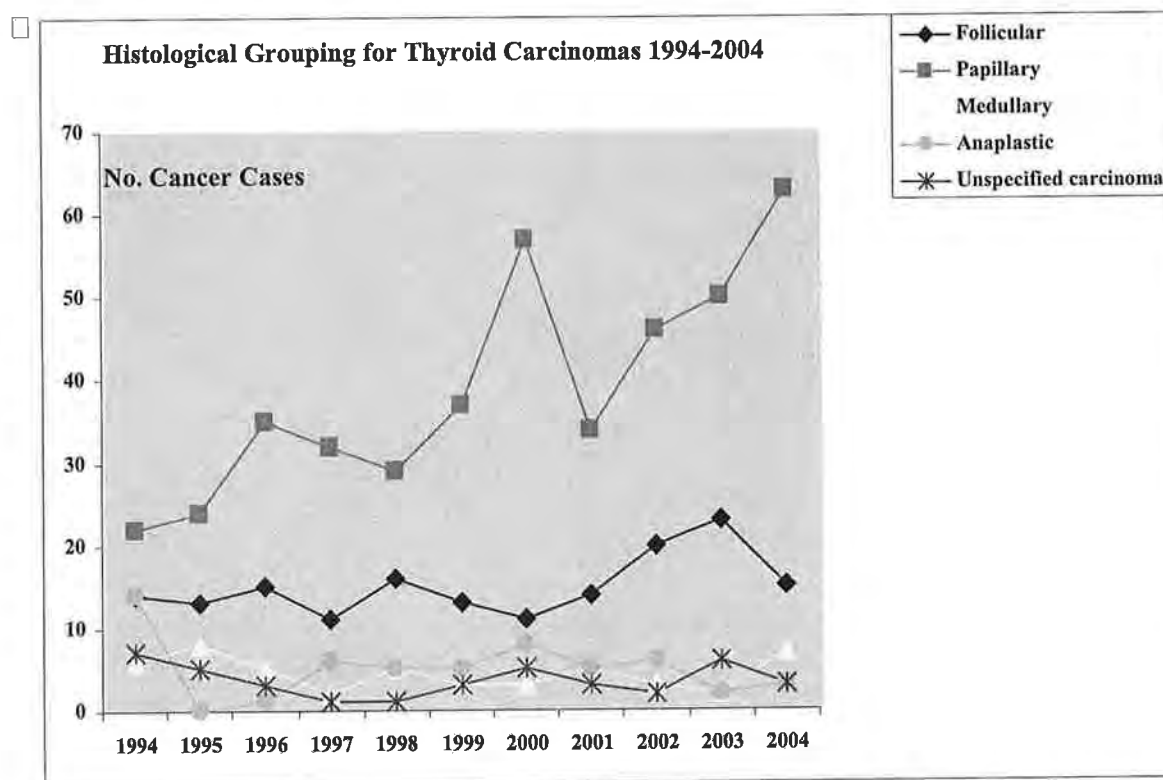


Fig 3.3.1a Incidence of thyroid cancers in the Republic of Ireland 1994-2004

This graphs demonstrates the incidence of thyroid cancer categorized by histological grouping for patients diagnosed in Ireland during the ten year period between 1994 to 2004.

The Irish statistics as analysed from our database also demonstrate an increase in the numbers of Papillary thyroid cancers. The Anaplastic figures are relatively constant over the time period with a total of 51 cases. According to the current system of classification of oncology disease, IARC have a category of "unspecified carcinoma" split into 'undifferentiated/anaplastic' and 'other'. Both of these subgroups include cancers with the same non specific morphology (eg M-8010 and M-8011) obviously using the grade to allocate them into anaplastic or not. Some of those extra or odd cases we have in the Irish system listed as 'Grade 4' have very specific morphologies eg M-8340/3 (papillary carcinoma follicular variant) - so it's debatable whether that can be classed as truly anaplastic?

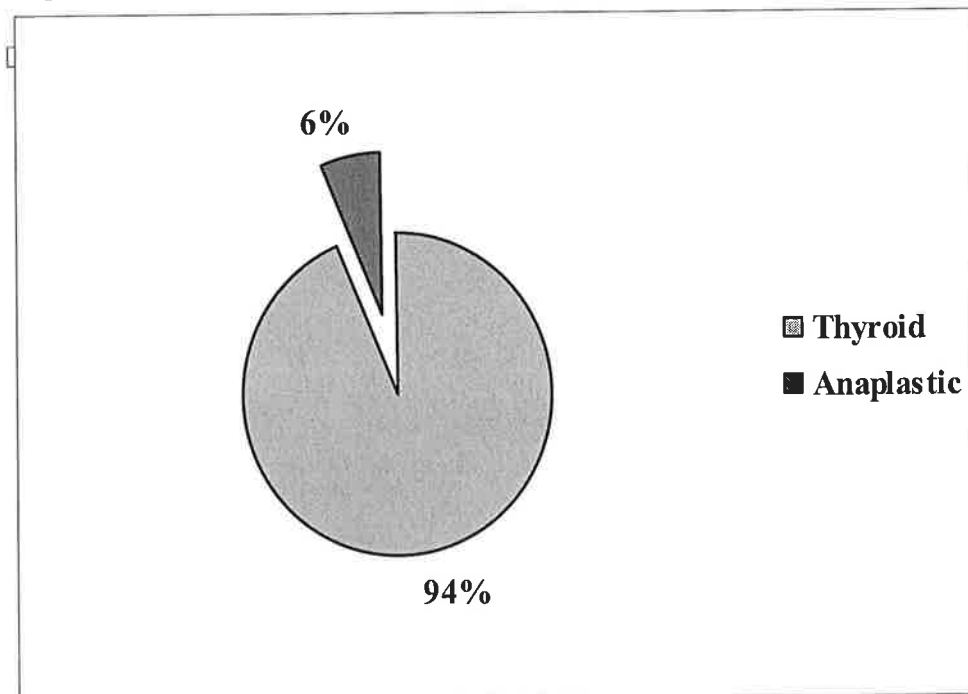


Fig 3.3.1b Incidence of ATC in Republic of Ireland 1994-2004.

The incidence of anaplastic thyroid cancer as a percentage of total thyroid cancer diagnosed in Ireland during the ten year period between 1994 to 2004.

We consulted with the data managers and other researchers here about the definition of anaplastic thyroids to be certain that we have extracted the cases correctly. We extracted the original dataset as well as the individual cases based on their morphologies matching that defined by the IARC - as published in their report "Histological groups for comparative studies" Parkin et al 1998 (IARC technical report no. 31. This seems to be the most precise definition. However having now looked at other thyroid morphologies (other than those included in the anaplastic range in the IARC report) that had a grade of IV we found there were a substantial number.

In total in the 11 year period, 1994-2004, there were 51 cases of ATC according to the IARC definition but an additional 27 cases if you were to include those 'other morphological types' grade IV. Most of these 27 cases were non specific morphologies eg M-8010/3 "carcinoma, NOS" which are classed as just unspecified carcinoma in the IARC manual.

In an unpublished manual in the National Registry's office "The Registrar's key to abstracting" compiled by April Fritz, Certified Tumour Registrar working in the National Cancer Institute in the US (2001) it's stated that, "undifferentiated carcinoma of the thyroid (including small cell or giant cell) comprises between 10 and 15% of all thyroid cancers.

This is an unpublished manual however it does reflect the impression we received from the National Registry that the current classification systems are not perfect but are internationally agreed. The statistics we quote are the cases on the database as defined by the IARC system of classification.

Given the surplus of cases that fall beyond these guidelines as identified to us by the National Registry suggests our figures for ATC are probably highly conservative.

3.3.2 Gender

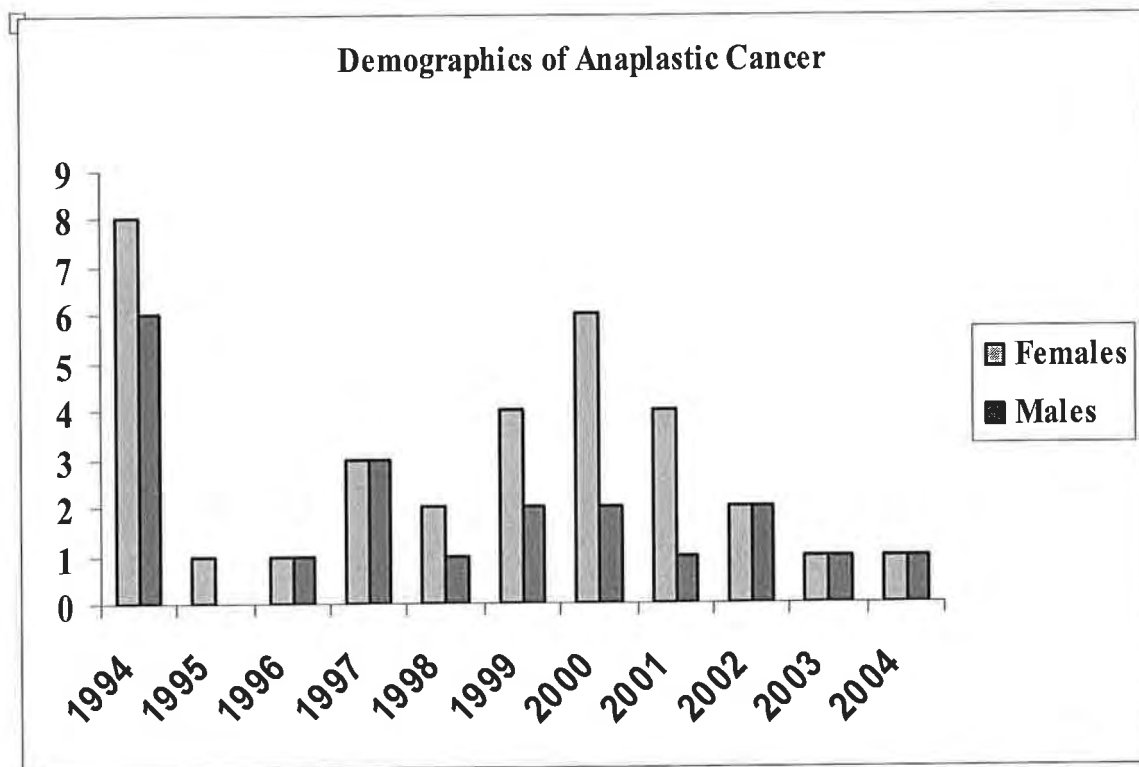


Fig 3.3.2a Incidence of ATC in the republic of Ireland according to gender 1994-2004.
The incidence of anaplastic thyroid cancer diagnosed in Ireland during the ten year period between 1994 to 2004 categorized by gender.

Factors associated with thyroid cancer include a family history of thyroid cancer, gender, (women have a higher incidence of thyroid cancer), age (the majority of cases occur in people over 40), although thyroid cancer affects all age groups from children through seniors, and prior exposure of the thyroid gland to radiation. Patients with a history of radiation administered in infancy and childhood for benign conditions of the head and neck, such as enlarged thymus, acne, or tonsillar or adenoidal enlargement, have an increased risk as well as other abnormalities of the thyroid gland. In this group of

patients, malignancies of the thyroid gland first appear beginning as early as 5 years following radiation and may appear 20 or more years later. Radiation exposure as a consequence of nuclear fallout has also been associated with a high risk of thyroid cancer, especially in children. Other risk factors for the development of thyroid cancer include a history of goiter, family history of thyroid disease, and Asian race.

Our figures reflect the gender predisposition involved in ATC and the majority of patients presenting being women (Fig 3.3.2a). This is what led to the development of our idea that an endocrine carcinogenesis or treatment strategy may play a role in this disease.

□3.3.3 Histopathology

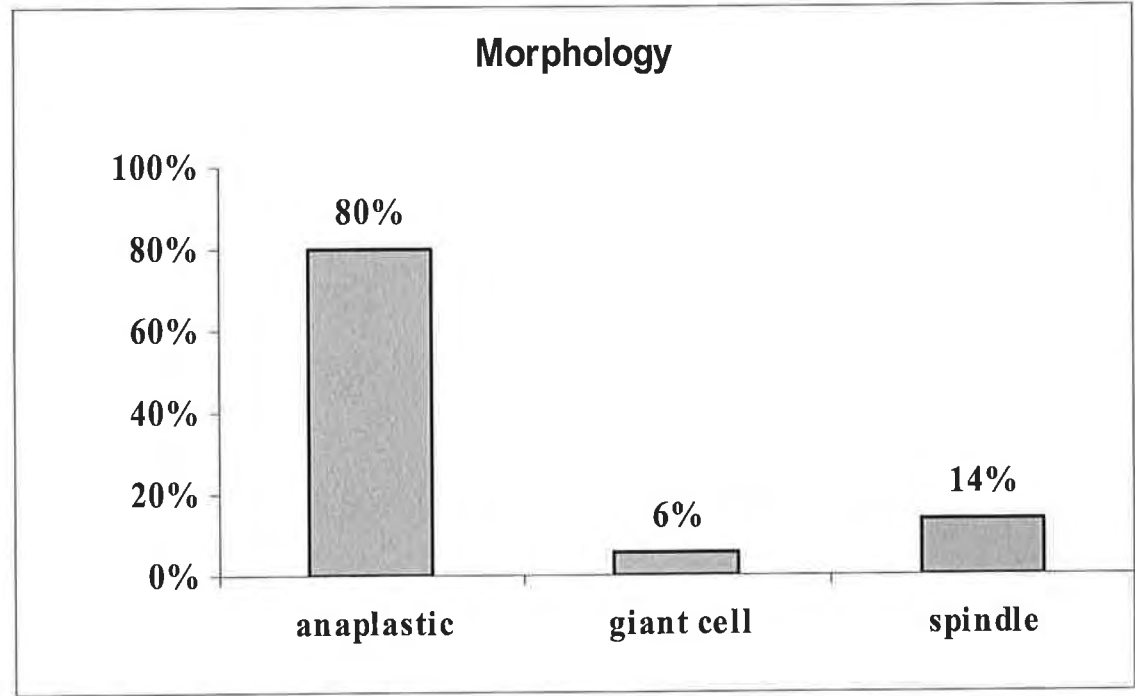


Fig 3.3.3 Histopathological breakdown of Anplastic thyroid cancer subtypes.
The percentage of anaplastic thyroid cancer diagnosed in Ireland during the ten year period between 1994 to 2004 categorized by morphology.

As we discussed in chapter 1 the pathogenesis of ATC is not completely understood. Whether it arises *de novo* or from a pre-existing well differentiated thyroid carcinoma (WDTC) is an area of controversy. The literature would reflect that it is probably both.

The progression of WDTC to ATC has been well documented at a clinical and molecular level with the loss of the *p53* tumor suppressor gene. Furthermore, coexistence of WDTC and ATC with zones of transition have been well described. Demeter et al (1991) found 76% of ATC had previous or concurrent thyroid disorders, with 47% related to WDTC. Some authors have suggested that all ATC contain foci of WDTC and that the inability to detect these foci is due to inadequate sectioning of the specimen (Volante et al 2008).

Papillary thyroid carcinoma is the most common type of thyroid cancer associated with ATC; biologically aggressive variants such as Tall cell are more common. Foci of Papillary Differentiated Thyroid Carcinoma (PDTC) are also common in ATC. Recent genetic studies have identified the *BRAF* mutation as the most common mutation leading to the formation of papillary thyroid cancer. Several studies have now shown that some ATCs may be derived from *BRAF*-mutated papillary thyroid cancer, and targeted expression of *BRAF* in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation (Cradic et al, 2009).

Anaplastic cells typically do not have thyrotropin receptors, do not transport iodine, and do not produce Thyroglobulin (TG). As with PDTC, more aggressive therapy may be warranted in patients with WDTC containing anaplastic foci. Figures from the database only reflected 14% of Irish ATC tumours being Spindle cell and 6% Giant cell. Future

attention to the anaplastic subtypes is necessary to reliably document the exact breakdown, and evaluate any differing clinical prognosis.

3.3.4 Survival

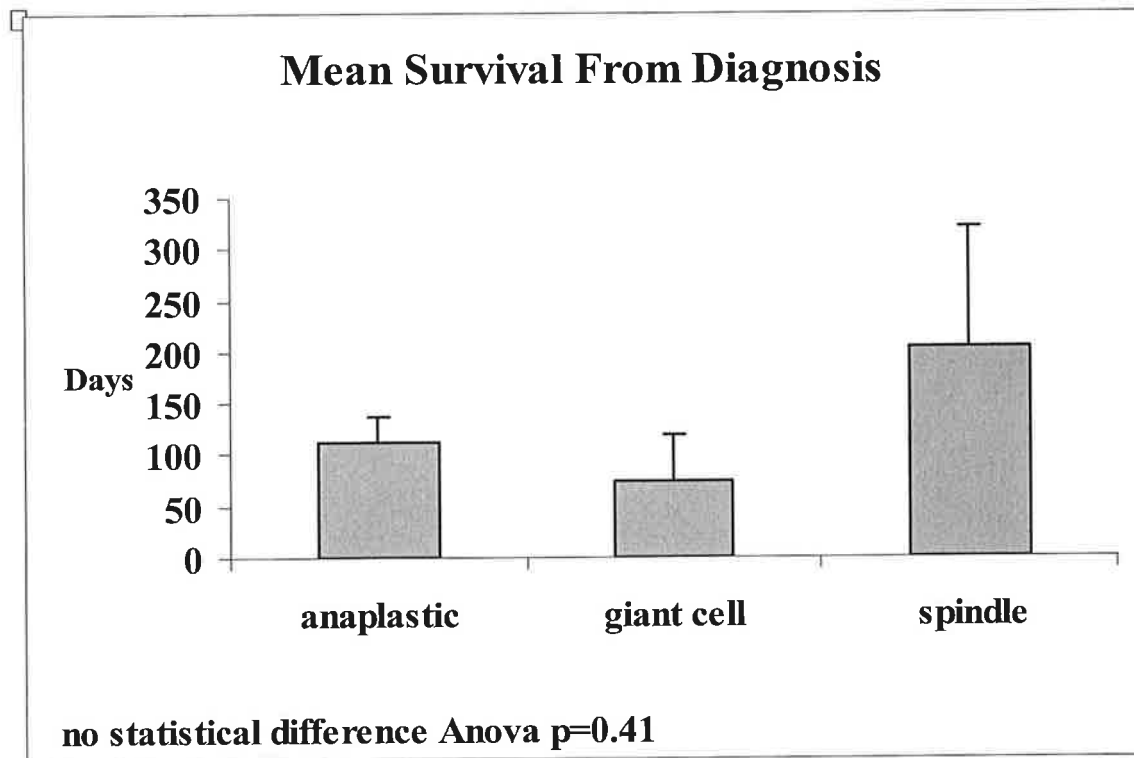


Fig 3.3.4 Mean survival from diagnosis in Republic of Ireland 1994-2004

This graph observes day's survival from diagnosis. Once the key histologies were separated we found no statistical difference in survival between them. This confirms although there are variation within the microscopic classification of the disease, pathologic subtypes have identical clinical behaviours and have no differing prognostic significance.

3.3.4.1 Mean survival from treatment

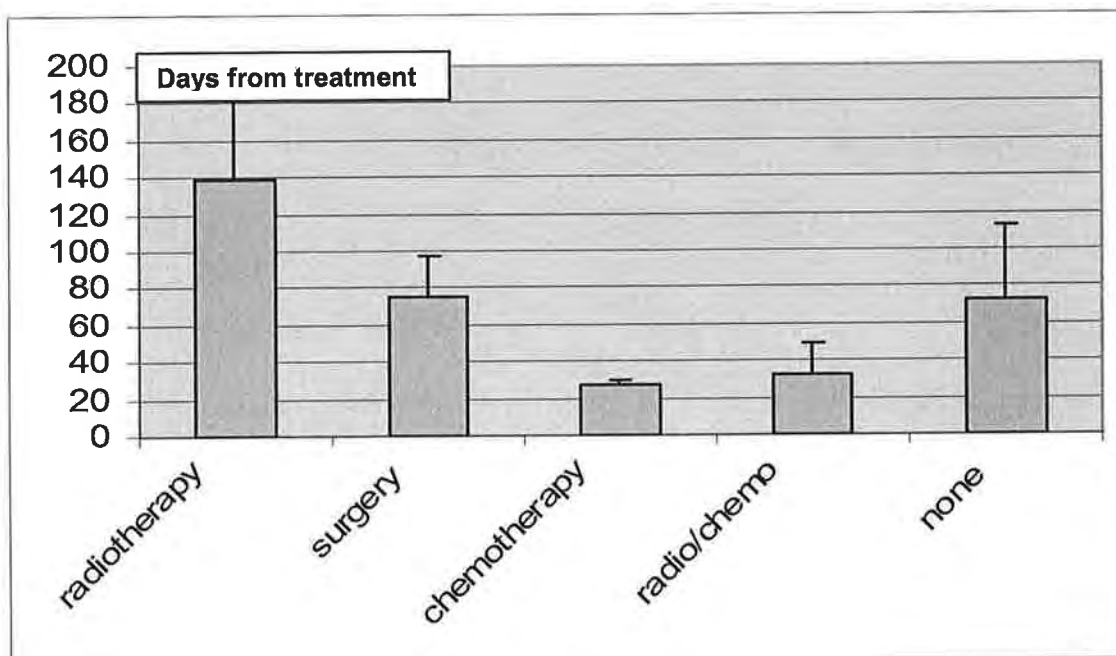


Fig 3.3.4.1 a Mean survival in day according to treatment type

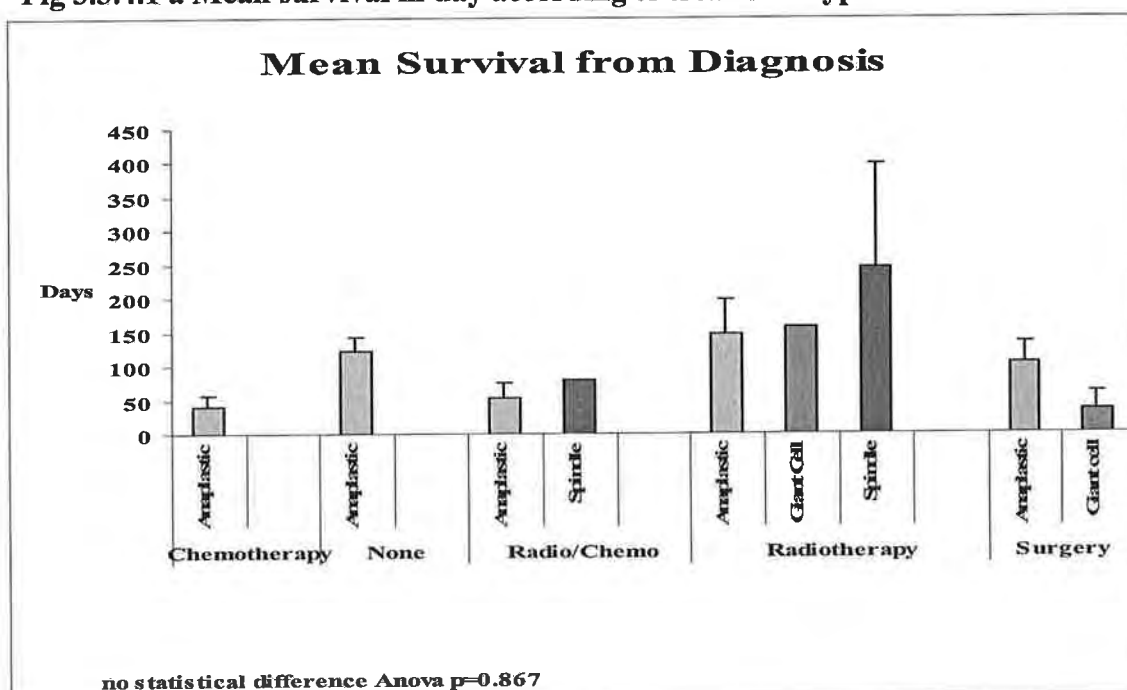


Fig 3.3.4.1 b Mean survival in Days from diagnosis by histology and treatment.

These graph demonstrate no significant difference in any treatment modality for anaplastic thyroid cancer. Furthermore fig (b) breakdown anaplastic thyroid cancer into the morphological subtypes revealing no improved survival for any treatment strategy currently employed.

Although rare, representing only 2-5% of clinically recognized thyroid cancers, the overall median survival is limited to months. Most patients are elderly and seek treatment with a rapidly growing mass. Almost half the patients seek treatment with distant metastases, with as many as 75% developing distant disease during their illness.

In most of the patients, complete surgical resection is not possible. There are, however, a few patients with resectable disease reported in the literatures who have demonstrated long-term survival with aggressive multimodal therapy that included surgery, radiation, and chemotherapy (Wiseman et al, 2007). Preclinical studies in human ATC cell lines show promise that new approaches to the management of this disease will be found in the future. Our figures currently show no survival advantage from adopting any therapy modality.

3.3.5 Smoking

Although tobacco and alcohol use are the main risk factors for most types of *head and neck cancer* (including cancers of the mouth, throat, and esophagus). Neither of these behaviours, however, has been shown to be risk factors for thyroid cancer. Nearly 50 % of patients presenting have never smoked.

One study recently questioned the negative association between thyroid cancer, whose etiology is largely uncertain with cigarette smoking. (Zivaljevic V et al 2004)

They examined the association between cigarette smoking and risk for female thyroid cancer. According to univariate analysis, female thyroid cancer was negatively associated with the initiation of smoking at a younger age, before the age of 20 (OR = 0.66, 95% CI = 0.50-0.90).

None of the smoking habits remained independently related to female thyroid cancer after adjustment for other factors which were significantly associated with thyroid cancer in the present study. The results of the study did not suggest a role of cigarette smoking in the development of thyroid cancer in women.

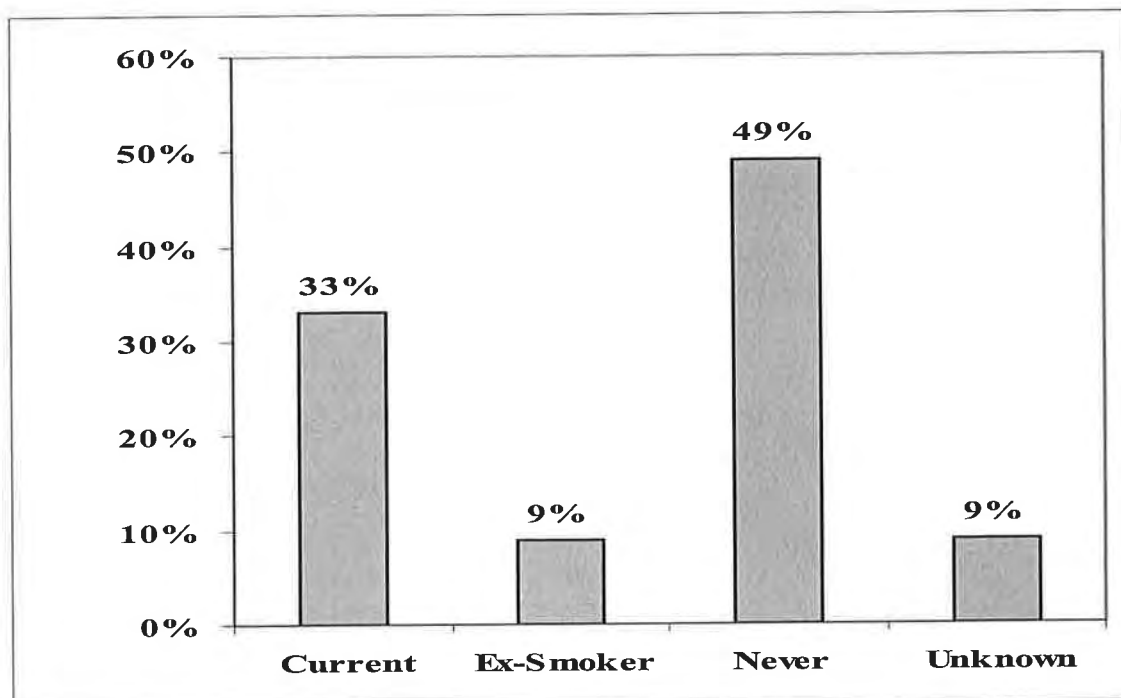


Fig 3.3.5a The percentage of presenting thyroid cancer patients who smoked.

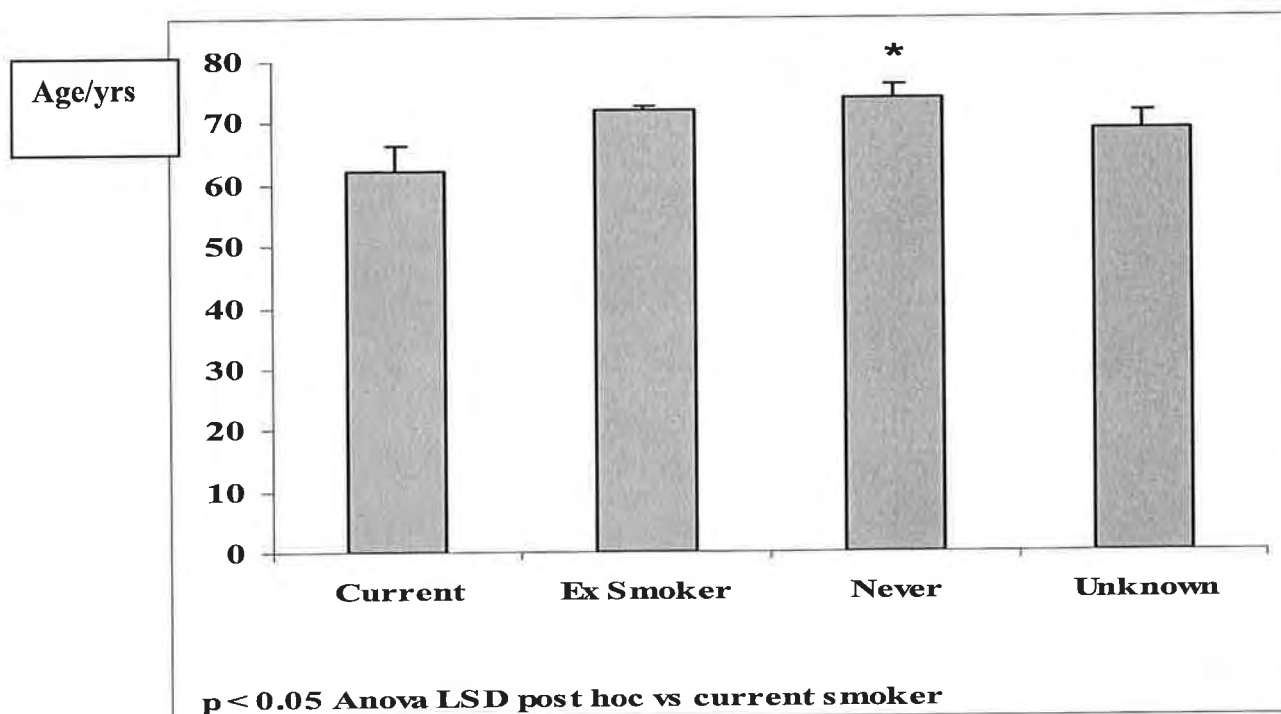
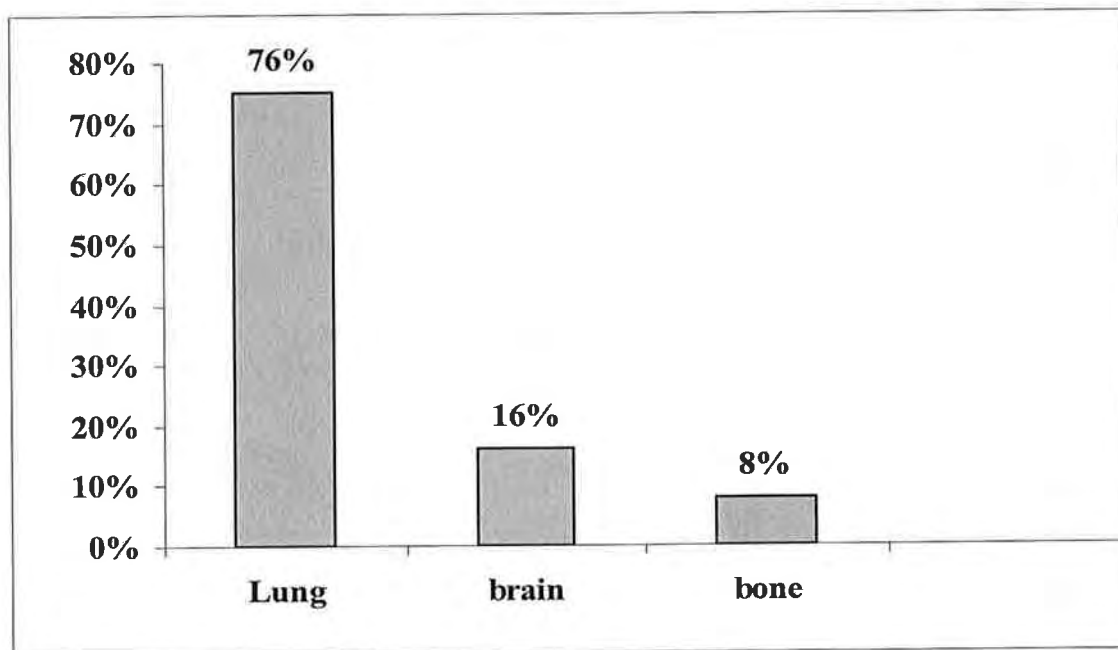


Fig 3.3.5 Age of presentation according to smoking habit 1994-2004.

The presentation in years of age of the patients and their smoking status is examined. ATC patients who did smoke presented on average one decade before the non smokers. We believe further analysis is required to investigate the effects of smoking and the possible implications on the post malignant de-differentiation.

3.3.6 Metastases (26%)



□ Fig 3.3.6 Site of distal metastases.

This graph demonstrates the site of metastatic deposits of ATC patients on presentation. In total 26% of patients on presentation had a metastatic deposit.

Anaplastic cancers invade adjacent structures and metastasize extensively to cervical lymph nodes and distant organs such as lung and bone. Tracheal invasion is present in 25% of patients at the time of presentation. This is why many patients with anaplastic thyroid cancer will need a tracheostomy while almost nobody with the other types of thyroid cancer will need one. Most of these cancers are so aggressively attached to vital neck structures on presentation that they are inoperable. Seventy six percent of the patients within our population had lung metastases at the time of diagnosis. Aggressive treatment protocols including hyperfractionated radiation therapy, chemotherapy, and surgery, fail to better 10% survival at 3 years.

3.4 Northern Ireland Statistics

The Northern Ireland Cancer office receive and store site codes according to ICD-10, and receive Snomed morphology codes from pathology. When a query comes in they use this information to furnish a reply. So for the Anaplastic Thyroid Cancer query they reviewed C73 ICD-10 and M80213 Snomed. This analysis was possible due to Finian Bannon at Queen's University, Belfast.

ICD-0 was formed using (and altering) ICD-10 codes to pinpoint site, and Snomed morphology codes to pinpoint morphology. An ICD-10 code combines both site and tumour behaviour. ICD-0 has better precision than ICD-10, where on occasion in ICD-10 two tumours in one site might not be distinguished even though their morphology is quite different. ICD-O is recommended for cancer registries over ICD-10. The Northern Ireland Cancer registry supply ICD10 with Snomed.

In total they had 515 cases of thyroid cancer. Of these 25 cases were ATC. This makes their percentage rate of Anplastic cancer at 5%. Unlike the South they have not recorded an increase in Papillary Cancer incidence increasing.

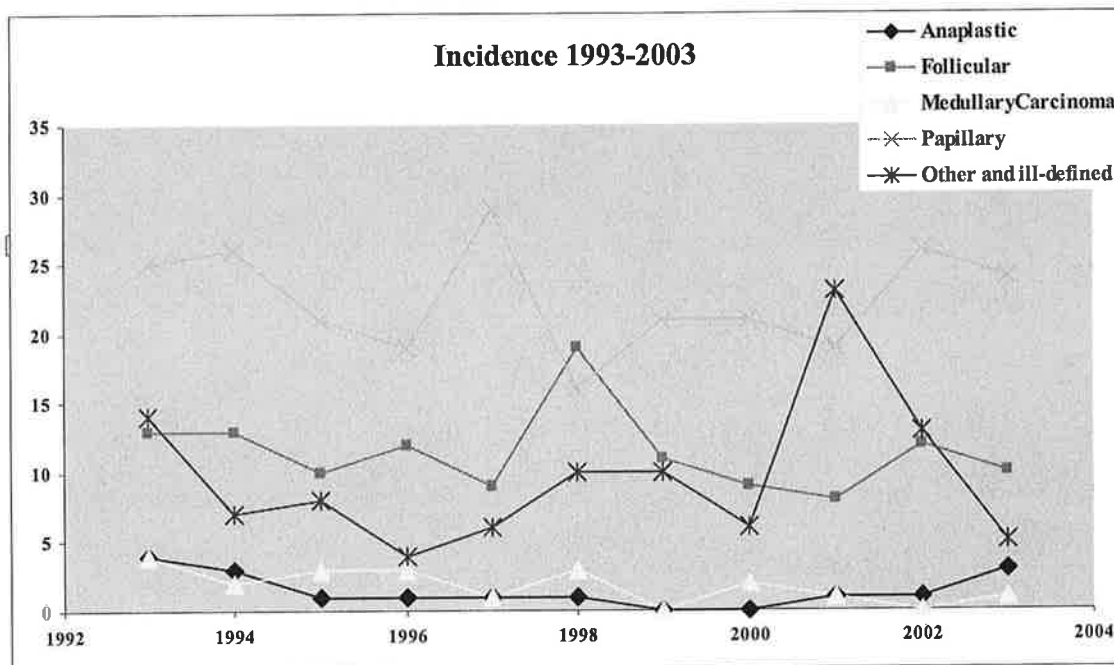


Fig 3.4a Incidence of ATC in Northern Ireland 1992-2004.

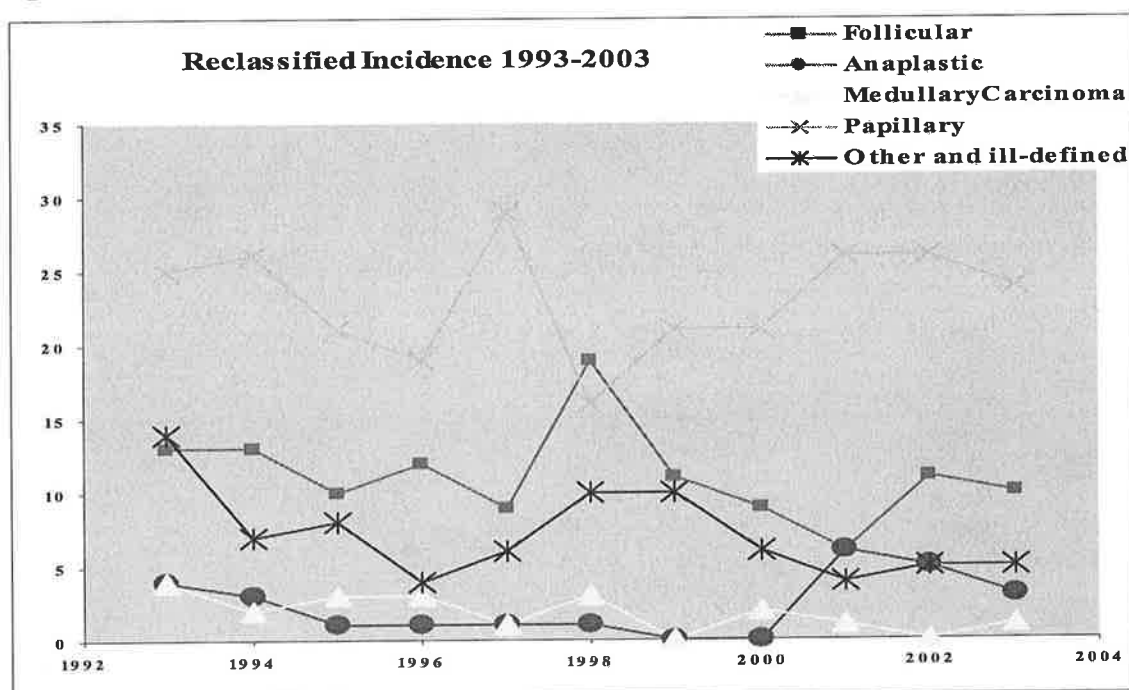


Fig 3.4b Reclassified Incidence of ATC in Northern Ireland 1992-2004.

In these graphs we can appreciate the breakdown of all Thyroid Malignancies. There are a relatively constant number of ATC however we can also see a rise in 'Non Specific' malignancies. Further investigation into the 'Non Specific' histologies revealed many were of the 'Anaplastic Thyroid' variant. This graph (b) was generated from an audit involving all clinical notes in hospitals searched to pick up more information on morphology than the routine information gathering processes. This gives an idea of the underestimation of the graph above with regard to anaplastic. However, the anaplastics may still be underestimated because older people may not get histological verification if it is felt they are too old for surgery.

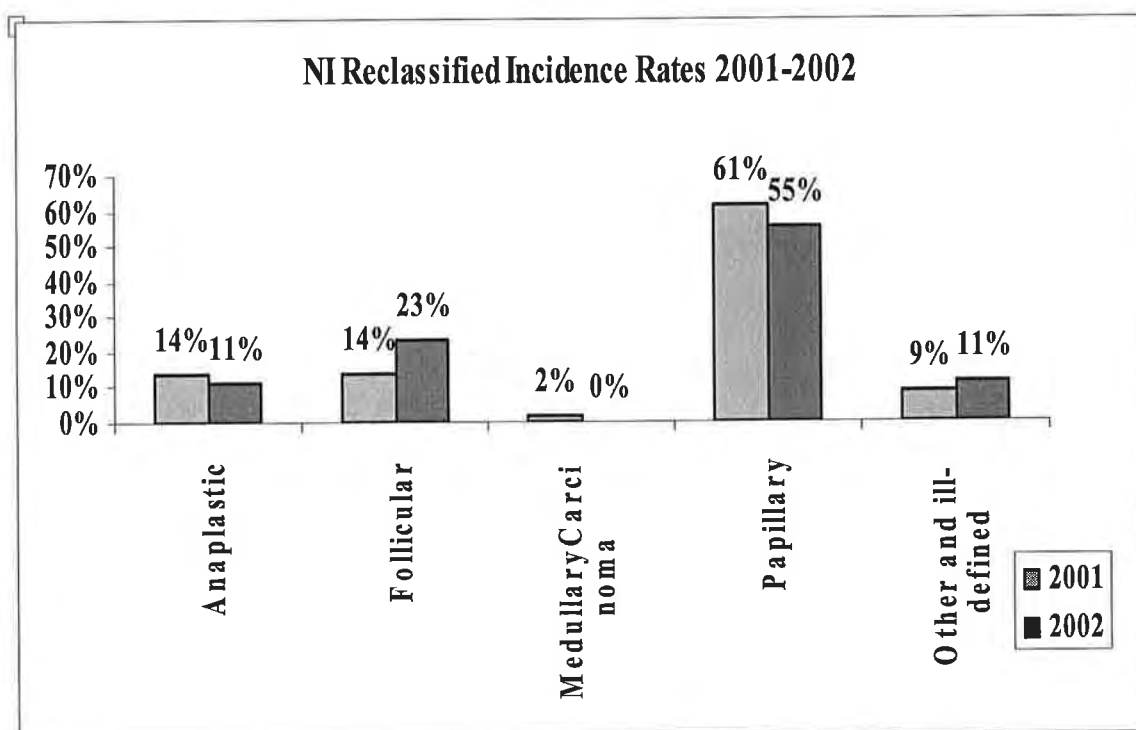


Fig 3.4c Reclassified Incidence of ATC in Northern Ireland 2001-2002.

This graph illustrates the findings of 2001 / 2002 following the audit taking place. This resulted in the overall Northern Irish Morphology changing to reveal a larger number of Anaplastic Cancers.

It also highlights the 27 cases in the South of Ireland which were labeled non specific morphologies eg M-8010/3 "carcinoma, NOS" which are classed as just unspecified carcinoma in the IARC manual. Our South of Ireland Statistics may indeed be highly conservative as we attempt to estimate this disease impact.

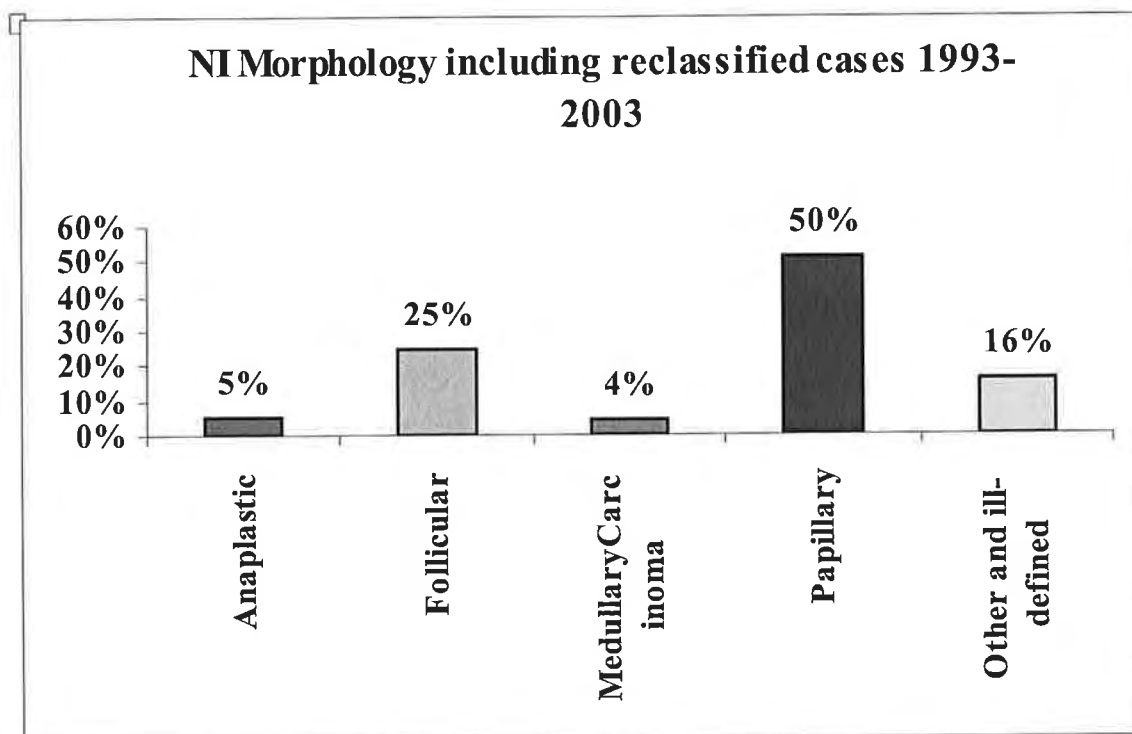


Fig 3.4d Reclassified Morphology of Thyroid Cancer in Northern Ireland 1993-2003.

This graph illustrates the findings of 1993-2003 audit. This resulted in the overall Northern Irish Morphology changing to reveal a larger number of Anaplastic Cancers.

In the UK the age standardised incidence rates have remained around 2 per 100,000 population, although there has been a small increase in male incidence rates and a larger increase from 2.4 to 3.4 per 100,000 in female incidence rates between 1993 and 2002

Investigation of a series of cancer data is very useful in helping to identify Cancer Control priorities and achievements. In the Republic of Ireland and Northern Ireland, cancer mortality data has been published in an essentially similar format in both countries.

This disease has had major implications for both regions. Examination of mortality and morbidity information from Ireland will provide insights about the evolving cancer patterns and provide necessary background to evaluate the impact of the cross-border cancer research activities being launched.

3.5 Epidemiology Conclusion

3.5.1 Global Cancer Conclusions:

The incidence of thyroid cancer increased from 3.6 per 100 000 in 1973 to 8.7 per 100 000 in 2002 - a 2.4-fold increase. There was no significant change in the incidence of the less common histological types: follicular, medullary, and anaplastic. Virtually the entire increase is attributable to an increase in incidence of papillary thyroid cancer, which increased from 2.7 to 7.7 per 100 000 - a 2.9-fold increase. Between 1988 (the first year SEER collected data on tumor size) and 2002, 49% of the increase consisted of cancers measuring 1 cm or smaller; 87% consisted of cancers measuring 2 cm or smaller. Mortality from thyroid cancer was stable between 1973 and 2002 (approximately 0.5 deaths per 100 000).

The increasing incidence of thyroid cancer in the United States is predominantly due to the increased detection of small papillary cancers. These trends, combined with the known existence of a substantial reservoir of sub clinical cancer and stable overall mortality, suggest that increasing incidence reflects increased detection of sub clinical disease, not an increase in the true occurrence of thyroid cancer.

3.6 Irish Head and Neck Oncology Database (IHNOD)

Continuing on from this work we have now established the Irish Head and Neck Oncology Database, IHNOD.

This National database is evaluating the main characteristics of the cancers presenting to Head and Neck Services around the country. The initiative for this database was made by Professor Michael Walsh at The Royal College of Surgeons in Ireland. Our epidemiology experiences from this chapter were employed to create IHNOD. We believe this represents progression of knowledge and an excellent example of collusion within our specialty.

In 2002, The British Association of Head and Neck Oncologists (BAHNO) agreed to join forces with partners to deliver a national comparative audit based upon the National Cancer Data Set (NCDS) subset for head and neck cancer.

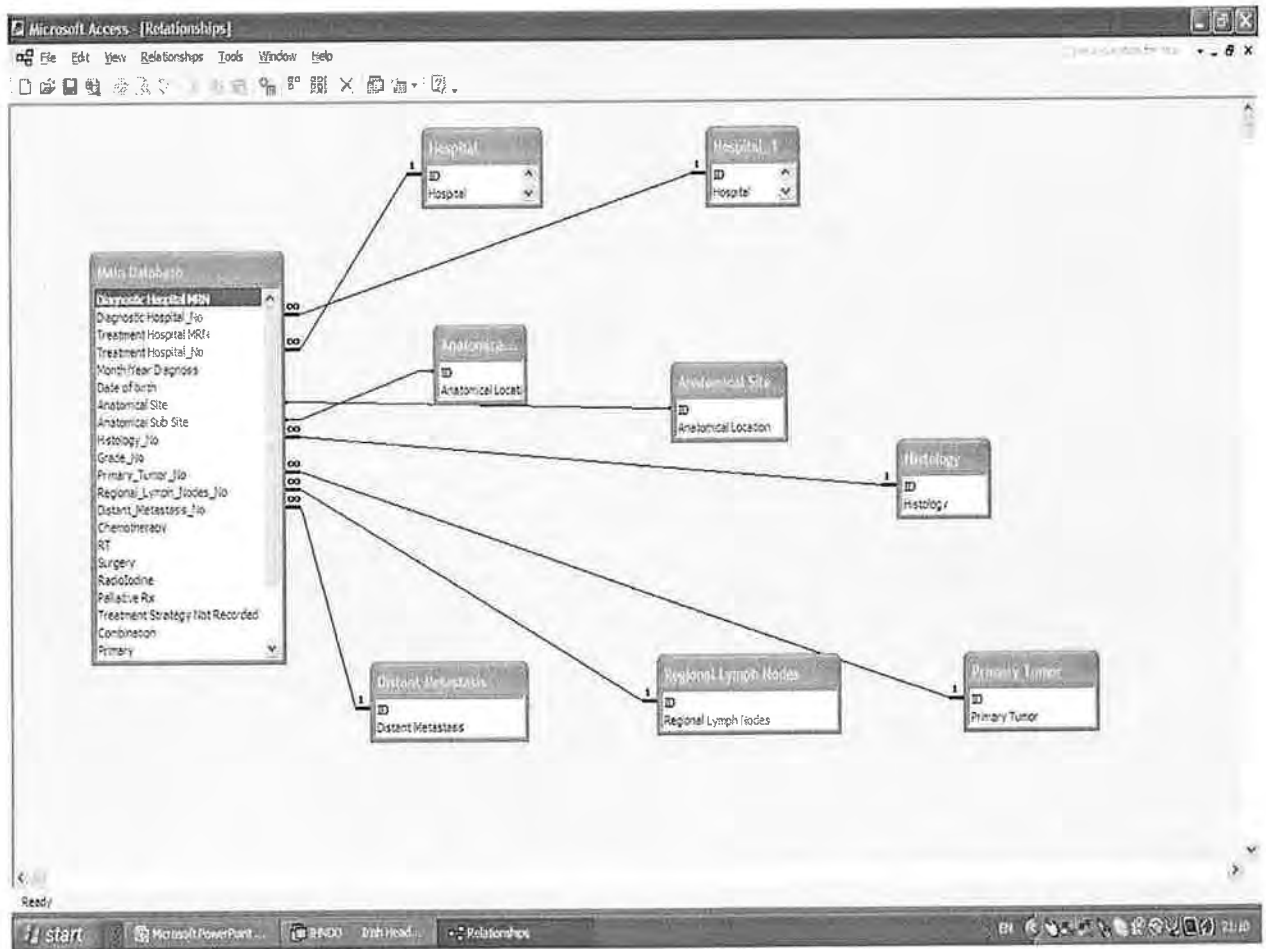
The formation of **DAHNO: Data for Head and Neck Oncology**, in 2002- England, and 2003- Wales, has provided both a technical infrastructure for data collection across these two countries as well as facilitating for local and central data analysis to deliver continuous comparative audit.

In an effort to evaluate our own patients, in cooperation and not in competition with the National Registry Database, we considered the DAHNO database. We have established a Microsoft Access database with all aspects of tumour features recorded.

3.6.1 The Fields of investigation are :

- 1. DIAGNOSTIC HOSPITAL**
- 2. TREATMENT HOSPITAL**
- 3. MONTH OF DIAGNOSIS**
- 4. AGE**
- 5. SEX**
- 6. TUMOUR LOCATION – BOTH ANATOMICAL REGIONS AND PRECISE ANATOMICAL ORIGIN**
- 7. HISTOPATHOLOGY ACCORDING TO AJCC CANCER STAGING HANDBOOK**
- 8. STAGING TNM CLASSIFICATION AND GRADE ACCORDING TO AJCC CANCER STAGING HANDBOOK**
- 9. TREATMENT OPTIONS EMPLOYED**
- 10. PRIMARY DISEASE OR RECURRENCE**
- 11. INTERVAL TIME PERIOD IF RECURRENCE**

We pre-programmed our database with all of the variables within each field. This was in an effort to reduce the manual entry of data which may lead to spelling and grammar mistakes. As a result the computer fails to link pieces of vital information. This is a global problem with the lesson being, the more you write, the more you devalue your database.



□

Fig 3.6a screenshot of the imputing fields in IHNOD

Using Microsoft Access we developed a relational database using the variables listed in 3.6.1

A relational database is a database that conforms to the relational model, and refers to a database's data and schema (the database's structure of how those data are arranged).

The term "relational database" is sometimes informally used to refer to a relational database management system, which is the software that is used to create a relational database.

Microsoft Access - [Main Database - Table]

File Edit View Insert Format Records Tools Window Help

Treatment Hospital No Month/Year Of Date of birth Anatomical Site Anatomical Sub Site Histology No Grade No Primary T Repo

University College Hospital	Jan/2008	20-10-1952	Pharynx	Tonsil	Pharyngeal, Squamous Cell Carcinoma	G2 - Moderately Differentiate	T1	N0
Waterford Regional	Apr/2008	14-11-1924	Lip and Oral Cavity	Oral Cavity	Lymphoma, B Cell	Not Recorded	Not Rec	Not R
University College Hospital	Feb/2008	08-04-1959	Larynx	Supraglottic	Larynx, Squamous cell carcinoma	G3 - Poorly Differentiated	T3	N1
Waterford Whitefield	Feb/2008	08-10-1943	Larynx	Glottic	Larynx, Squamous cell carcinoma	G2 - Moderately Differentiate	T3	N0
Limerick Regional	Feb/2008	23-06-1963	Lip and Oral Cavity	Ant 2/3 Tongue	Lip and Oral, Squamous Cell Carcinoma	Not Recorded	T2	N2
Water	Jan/2008	20-09-1954	Pharynx	Base of Tongue	Pharyngeal, Squamous Cell Carcinoma	G3 - Poorly Differentiated	T2	N1
Waterford Regional	May/2008	12-01-1941	Pharynx	Pharynx	Pharyngeal, Squamous Cell Carcinoma	Not Recorded	T4	N2
Water	Feb/2008	11-12-1963	Other	Neck	Germ Cell	Not Recorded	Not Rec	N0
Royal Victoria Eye and Ear	Jan/2008	17-09-1931	Larynx	Glottic	Larynx, Verrucous Carcinoma	G1 - Well Differentiated	T1	N0
Limerick Regional	Jan/2008	26-08-1937	Nasal Cavity and Pa	Maxillary Sinus	Nasal and Paranasal Sinus, Squamous cell carc	G3 - Poorly Differentiated	T4	N0
Limerick Regional	Feb/2008	08-05-1922	Carcinoma of the St	Skin	Skin, Squamous Cell Carcinoma	G2 - Moderately Differentiate	T1	N0
Water	Feb/2008	30-07-1967	Melanoma of the St	Skin	Skin, Melanoma	Not Recorded	Not Rec	Not R
Limerick Regional	Feb/2008	04-10-1939	Larynx	Glottic	Larynx, Squamous cell carcinoma	G3 - Poorly Differentiated	T1	N0
Waterford Whitefield	Feb/2008	20-04-1926	Lip and Oral Cavity	Lip	Lip and Oral, Squamous Cell Carcinoma	G3 - Poorly Differentiated	T2	N0
St James	Apr/2008	29-05-1942	Pharynx	Pharynx	Pharyngeal, Squamous Cell Carcinoma	Not Recorded	T4	N2
Waterford Regional	Apr/2008	01-12-1934	Lip and Oral Cavity	Lip	Lip and Oral, Squamous Cell Carcinoma	Not Recorded	T2	N0
University College Hospital	Jan/2008	08-07-1933	Other	Neck	Squamous Cell Carcinoma, Unknown Primary	G3 - Poorly Differentiated	Tx	N2b
St Vincents	Feb/2008	08-10-1934	Larynx	Larynx	Larynx, Squamous cell carcinoma	Not Recorded	Not Rec	N2c
Pending	Feb/2008	30-11-1954	Larynx	Glottic	Larynx, Squamous cell carcinoma	G2 - Moderately Differentiate	T4	N1
St James	Mar/2008	03-04-1922	Pharynx	Oropharynx	Pharyngeal, Squamous Cell Carcinoma	Not Recorded	Not Rec	Not R
St Vincents	Feb/2008	11-02-1943	Carcinoma of the St	Skin	Skin, Basal Cell Carcinoma	Not Recorded	Not Rec	N2c
University College Hospital	Apr/2008	28-09-1959	Larynx	Glottic	Larynx, Squamous cell carcinoma	G2 - Moderately Differentiate	T1	N0
Limerick Regional	Feb/2008	14-05-1914	Carcinoma of the St	Pinna	Ear, Squamous Cell Carcinoma	G2 - Moderately Differentiate	T2	N2
University College Hospital	Apr/2008	09-06-1967	Lymphoid Neoplasm	Neck	Lymphoma, Nodular Sclerosis	Not Recorded	Not Rec	Not R
Waterford Regional	Feb/2008	21-07-1928	Other	Neck	Squamous Cell Carcinoma	Not Recorded	Tx	N1
Water	Jan/2008	28-02-1952	Pharynx	Tonsil	Pharyngeal, Squamous Cell Carcinoma	G2 - Moderately Differentiate	T1	N2c
University College Hospital	Jan/2008	23-07-1931	Pharynx	Base of Tongue	Pharyngeal, Squamous Cell Carcinoma	G3 - Poorly Differentiated	T1	N0
University College Hospital	Mar/2008	18-10-1947	Pharynx	Hypopharynx	Pharyngeal, Squamous Cell Carcinoma	Not Recorded	T2	N0
St Vincents	Apr/2008	24-07-1966	Larynx	Glottic	Larynx, Squamous cell carcinoma	Not Recorded	T1	N0
St Vincents	Apr/2008	02-04-1938	Larynx	Larynx	Larynx, Squamous cell carcinoma	G2 - Moderately Differentiate	T4	N0
University College Hospital	Feb/2008	10-11-1931	Lymphoid Neoplasm	Neck	Lymphoma, Mantle Cell	Not Recorded	Not Rec	Not R
University College Hospital	Mar/2008	30-11-1939	Pharynx	Tonsil	Pharyngeal, Squamous Cell Carcinoma	Not Recorded	T2	N2b
Royal Victoria Eye and Ear	Apr/2008	27-02-1952	Larynx	Larynx	Larynx, Squamous cell carcinoma	G2 - Moderately Differentiate	T4	N1
Water	Jan/2008	17-12-1911	Thyroid	Thyroid	Thyroid, Anaplastic	G4 - Undifferentiated	T4	N0
Water	Jan/2008	24-05-1950	Larynx	Glottic	Larynx, Verrucous Carcinoma	Not Recorded	T2	N0

Records: 14 of 170

Database View

Start Microsoft PowerPoint... IRKO Irish Health... Main Database - Table

Fig 3.6b screenshot of data entry in IHNOD

This screenshot is a sample of part of the IHNOD database now registered.

This Database marks a commitment to improved audit evaluation of our patients which we believe will impact on administrative, medical and surgical aspects of care. As of yet there is no comprehensive mapping of Head and Neck Oncology care delivered in Ireland.

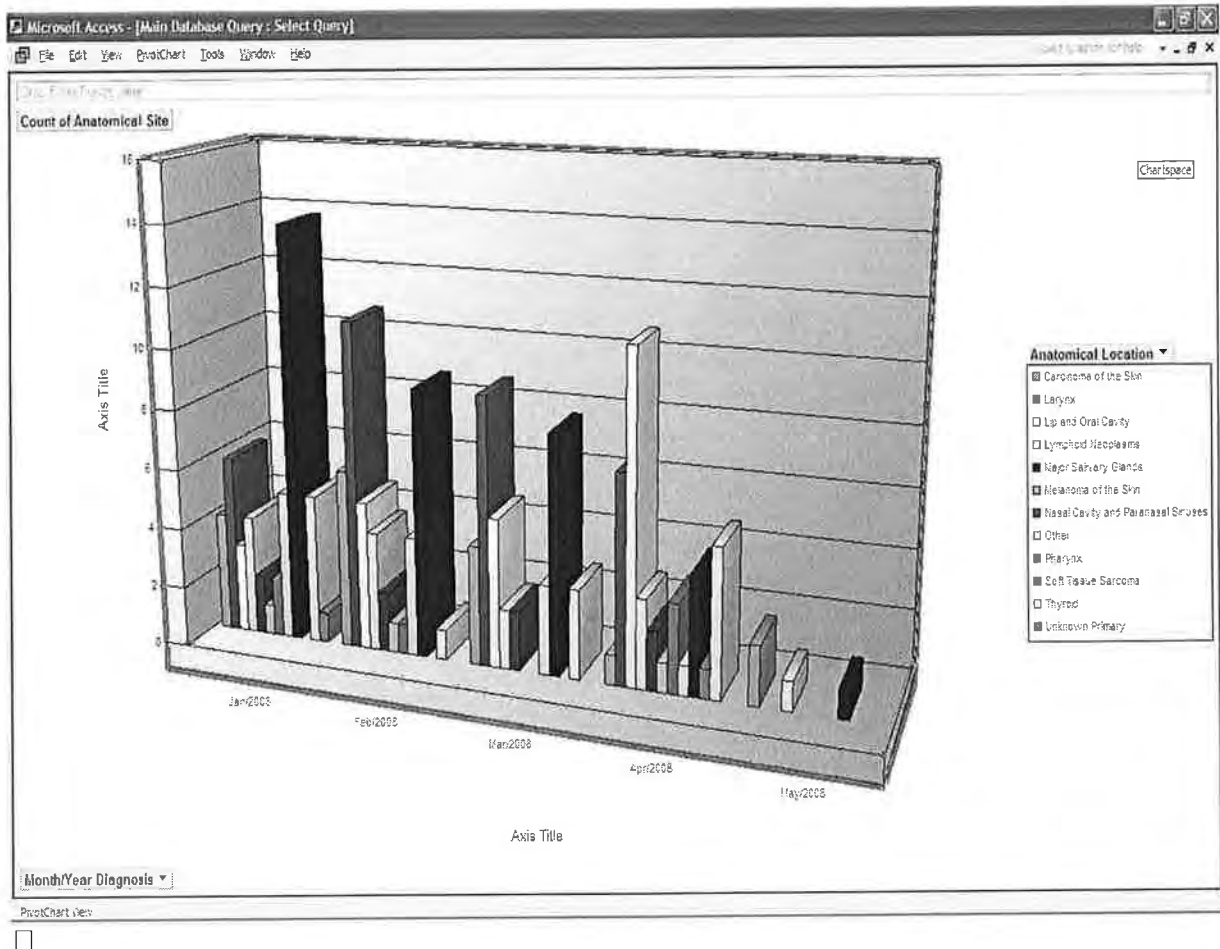


Fig 3.6c screencapture of data analysis possible in IHNOD

This Graph reports the locations of Cancers identified within the Head and Neck anatomical region.

IHNOD aspires to provide an electronic comparative audit. The immediate availability of accurate and timely data will provide Head and Neck Cancer teams and networks with the ability to accurately assess their strengths and weaknesses and to plan for more effective care.

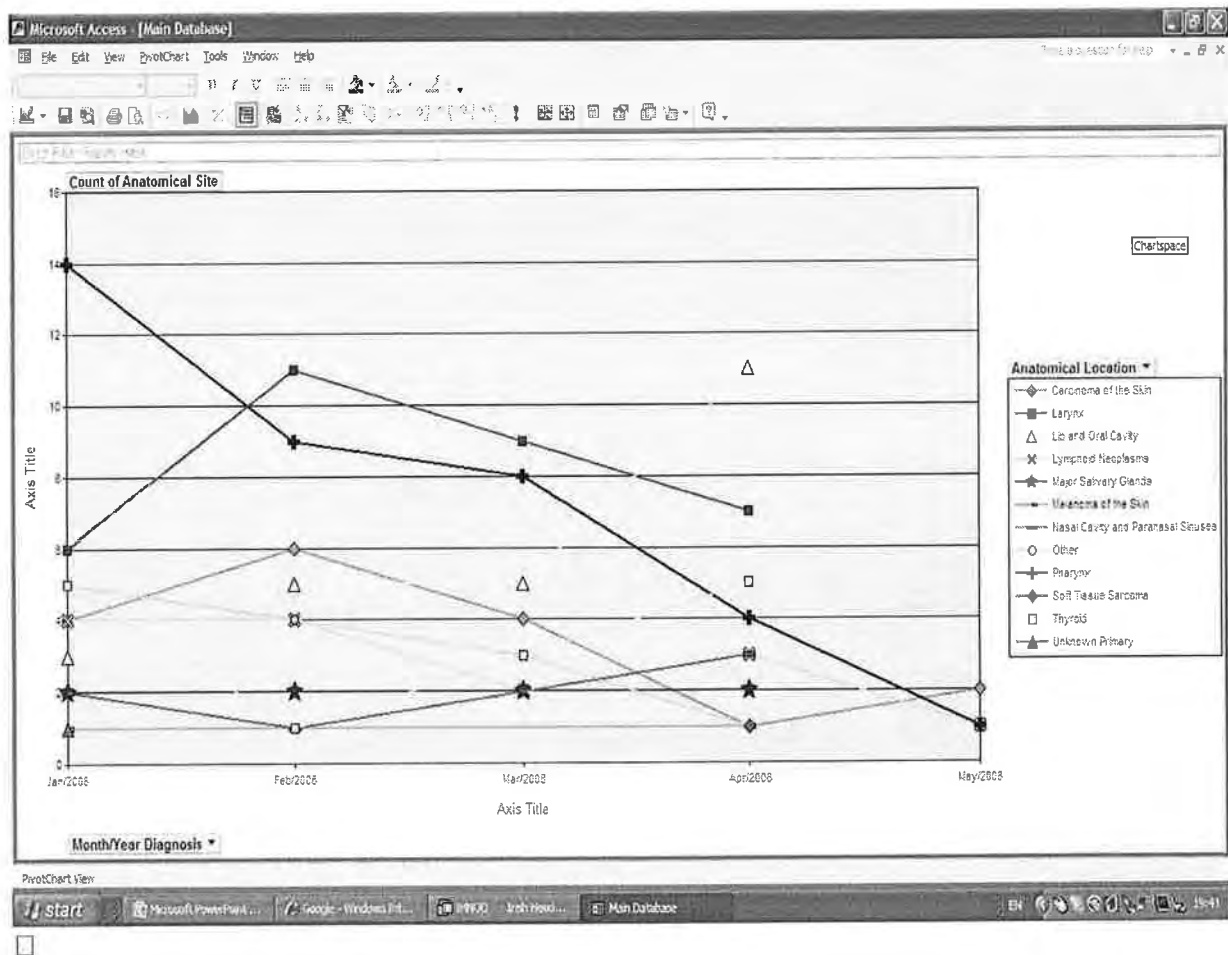


Fig 3.6d **Screencapture of data analysis in IHNOD Month of diagnosis Vs Anatomical location. This data is of particular use for audit presentation and has been presented at the, National ENT Grand Rounds**

The immediate response to this initiative in Ireland has been very positive. Over 95% of Irish Hospitals are now contributing their Head and Neck figures on a monthly basis and we envisage the first official publication of the figures in January 2009.

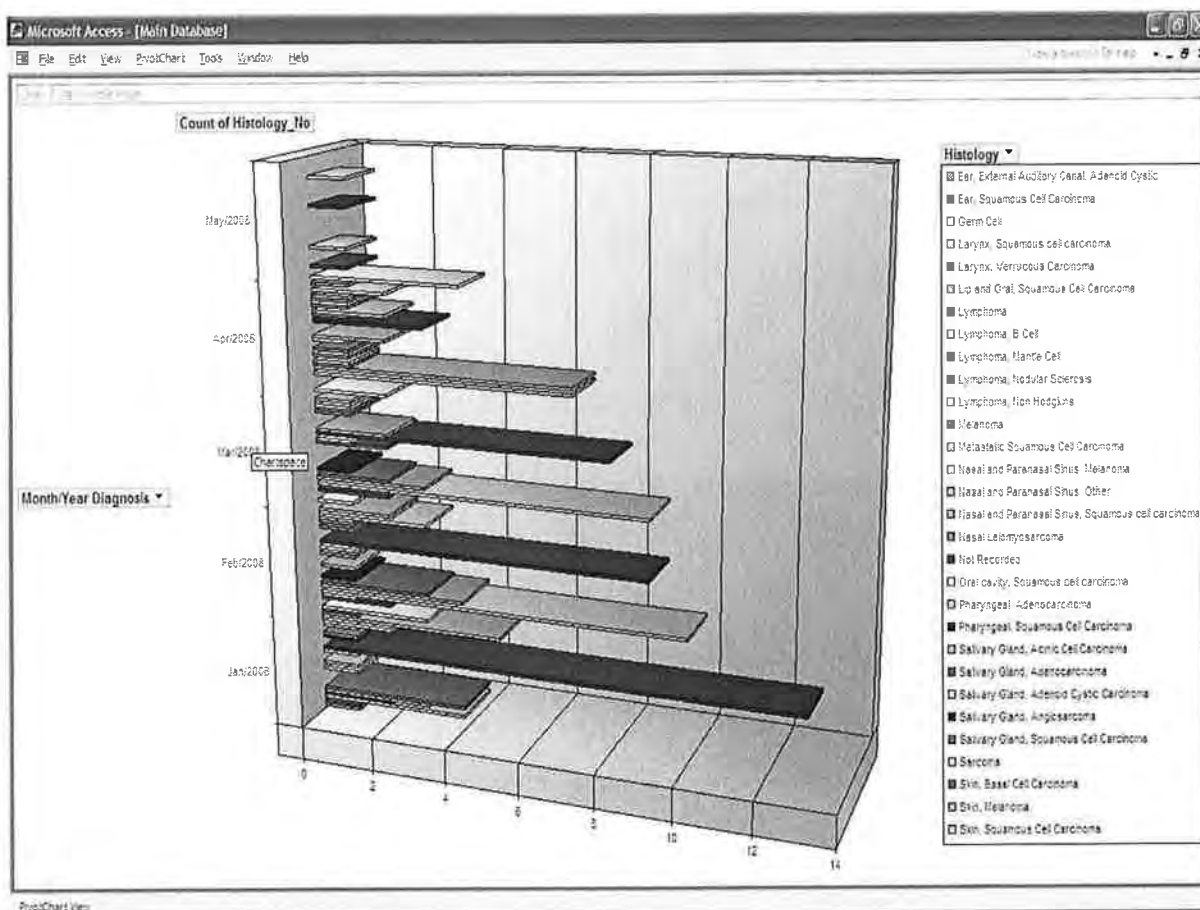


Fig 3.6e Screencapture of data analysis in IHNOD Month of diagnosis Vs Histology. This graph outlines the histopathological breakdown of the Cancers documented (as of May 2008).

3.7 Irish Perspective: Audit ATC Key points

The main points from this audit were as follows. Higher levels of this disease are shown compared to international statistics. Patient numbers may be higher due to the shortfalls in the coding and morphological system. A total of 812 cases of Thyroid Cancer were recorded in The Republic of Ireland with 51 cases of ATC. This represents 6.28% of diagnosed thyroid cancers.

Northern Ireland had 515 cases with 25 cases of Anaplastic Thyroid Cancer which represents 4.85%. In Ireland we have therefore identified this disease as being a more common pathology than Medullary Thyroid cancer.

ATC makes a significant contribution to annual cancer mortality however no current treatment strategy offers improved statistical survival. Histopathological patterns of ATC cancer carry equal prognosis.

Over 50% of the patients within the Southern Ireland population never smoked however smoking encourages early presentation with smokers presenting a decade earlier. This is the first time this observation has been documented. ATC also has a female gender bias.

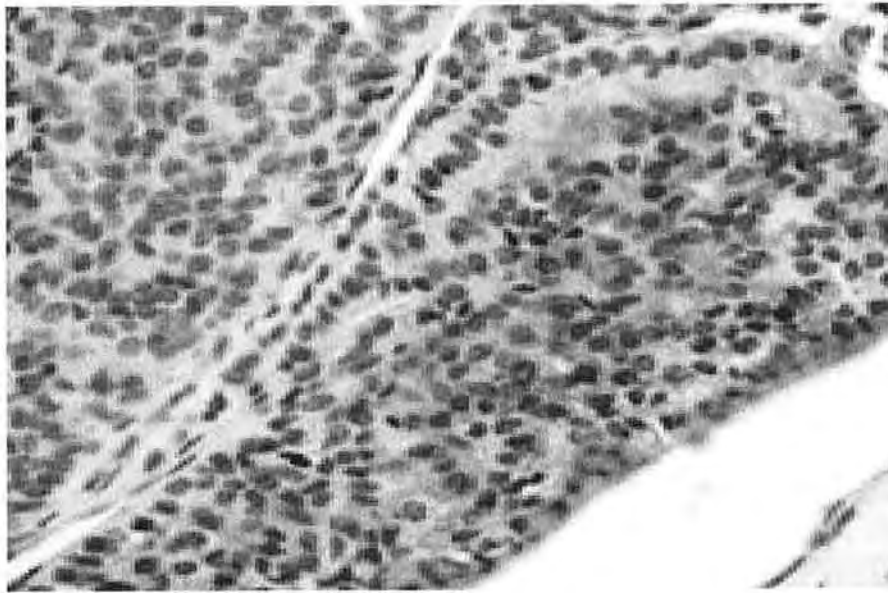
This indicates an endocrine related carcinogenic pathway and therapeutic strategy may be implicated. We now observe ATC tissue immunohistochemical properties and the role, if any, of the estrogen receptor.

A biproduct of this chapter has been the formation of The Irish Head and Neck Oncology Database representing a real commitment to Head and Neck Epidemiology and Management in Ireland.

Chapter Four

Immunohistochemical Analysis of

Human Anaplastic Thyroid Cancer Tissue



*Immunohistochemical staining of human thyroid gland cancer
with anti-ERK-1 antibody*

4.1 Introduction

Anaplastic thyroid carcinoma is one of the most aggressive human malignancies, with a median survival of up to 6 months. Currently no definitive therapeutic strategy exists for the majority of patients apart from a palliative course. This is due to the fact that 90% of patients have adjacent tissue invasion and 20-50% of patients have disseminated disease on presentation. The growing incidence of ATC coupled with such dire prognosis under the present treatment procedures advocates the need for novel approaches in the management of this disease. The previous chapter highlighted a growing female gender association with ATC thus implicating an endocrine carcinogenic pathway in the pathogenesis of this disease. Little is currently known regarding the expression by anaplastic tumors of molecular targets for new human anticancer agents that have been studied in the preclinical or clinical setting.

Her-2/neu is notable for its role in the pathogenesis of breast cancer and as a target for treatment. Approximately 25-30% of breast cancers have an amplification of the Her-2 or neu gene or overexpression of its protein product. Overexpression of this receptor in breast cancer is associated with increased disease recurrence and worse prognosis. Because of its prognostic role as well as its ability to predict response to trastuzumab, breast tumors are routinely checked for overexpression of Her-2/neu. Overexpression also occurs in other cancer such as ovarian cancer and stomach cancer. Clinically, Her-2/neu is important as the target of the monoclonal antibody trastuzumab (marketed as Herceptin). Trastuzumab is only effective in breast cancer where the Her-2/neu receptor is overexpressed. Her-2 is co-localized, and thus most of the time co-amplified with the gene GRB -7, which is also a proto-oncogene (active in e.g. breast cancer, testicular germ cell tumour, gastric cancer, and esophageal cancer).

The ErbB protein family or epidermal growth factor receptor (EGFR) family is a family of four structurally related tyrosine kinase receptors. The second member of this family is the Her-2 receptor.

There are two basic approaches to the development of drugs that inhibit epidermal growth factor receptors. Monoclonal antibodies (MAb) compete with growth factors to bind to the external receptor, thus blocking dimerization and activation of the tyrosine kinase enzyme. Cetuximab (trade name Erbitux) is approved by the FDA for use in advanced colorectal cancer. Clinical trials have indicated that cetuximab can enhance the antitumor effects of the chemotherapeutic drug cisplatin in head and neck cancer. This chimeric MAb derived from mice when used on a long-term basis, stimulates an antibody response by the patient's immune system and is destroyed by the body before it can exert an anti-cancer effect. A second generation humanized MAb, Panitumumab, is now in advanced clinical trials, and should receive FDA approval for marketing soon. Small-molecule drugs that inhibit activation of tyrosine kinase, thereby blocking the signaling process from the receptor is the second approach. Gefitinib (trade name Iressa) is currently on the market to treat advanced non-small cell lung cancer. The drug is primarily used as a "second-line" treatment after several treatments with chemotherapy have failed to stop progression of the disease. Studies are currently underway to evaluate gefitinib to treat other solid tumors, such as cancers of the breast, and colon and rectum. Again, most studies involve using gefitinib in combination with chemotherapeutic drugs. In the case of breast cancer, gefitinib appears to prolong the effectiveness of tamoxifen by blocking certain signaling pathways. Caponigro et al 2004 have also reported encouraging early data of Gefitinib in the treatment of Head and Neck Cancers.

Little data is currently available concerning receptor expression in anaplastic thyroid carcinomas. The objective of this work was to evaluate the expression profile of human anaplastic thyroid tumors for molecular targets for treatment particularly the oestrogen receptor, Her-2 and EGFR.

4.2 Methods

The Her-2/neu, ER-1, and EGFR expression in anaplastic thyroid cancer was investigated retrospectively using archival tissue from eight patients who attended St Vincents Hospital Dublin over a five year period from 1995 – 2005. Ethics approval was received from the St Vincent's Hospital Ethics committee. The Her-2, ER-1, and EGFR expression was immunohistochemically detected on sections from formalin-fixed, paraffin-embedded tissues using monoclonal antibody staining with Trilogy antigen retrieval and The Vector Elite Detection system to visualise the antibody-antigen complex. Our controls were ER-1 positive human breast tissue, HER positive human breast tissue and human placenta for erb-1. (See 2.10). ER-(was measured using Rabbit monoclonal antibody (clone SP1) supplied by Lab Vision (RM-9101-S). At the time this work was carried out ER-(was the clinically relevant receptor in relation to medicating tamoxifen's effects on cell function. Furthermore no reliable ER-(antibody was available for staining paraffin embedded tissue.

4.3 Results

Of the 8 ATC patient samples analysed 7 (87.5%) stained negative for ER-1 and ERB-1. 1 sample stained positive for both ER-1 and ERB-1. (Fig 4.1 & 4.2) 7 samples (87.5%) stained negative for Her-2 and one sample (a different sample from above) stained positive for Her-2. (Fig 4.3).

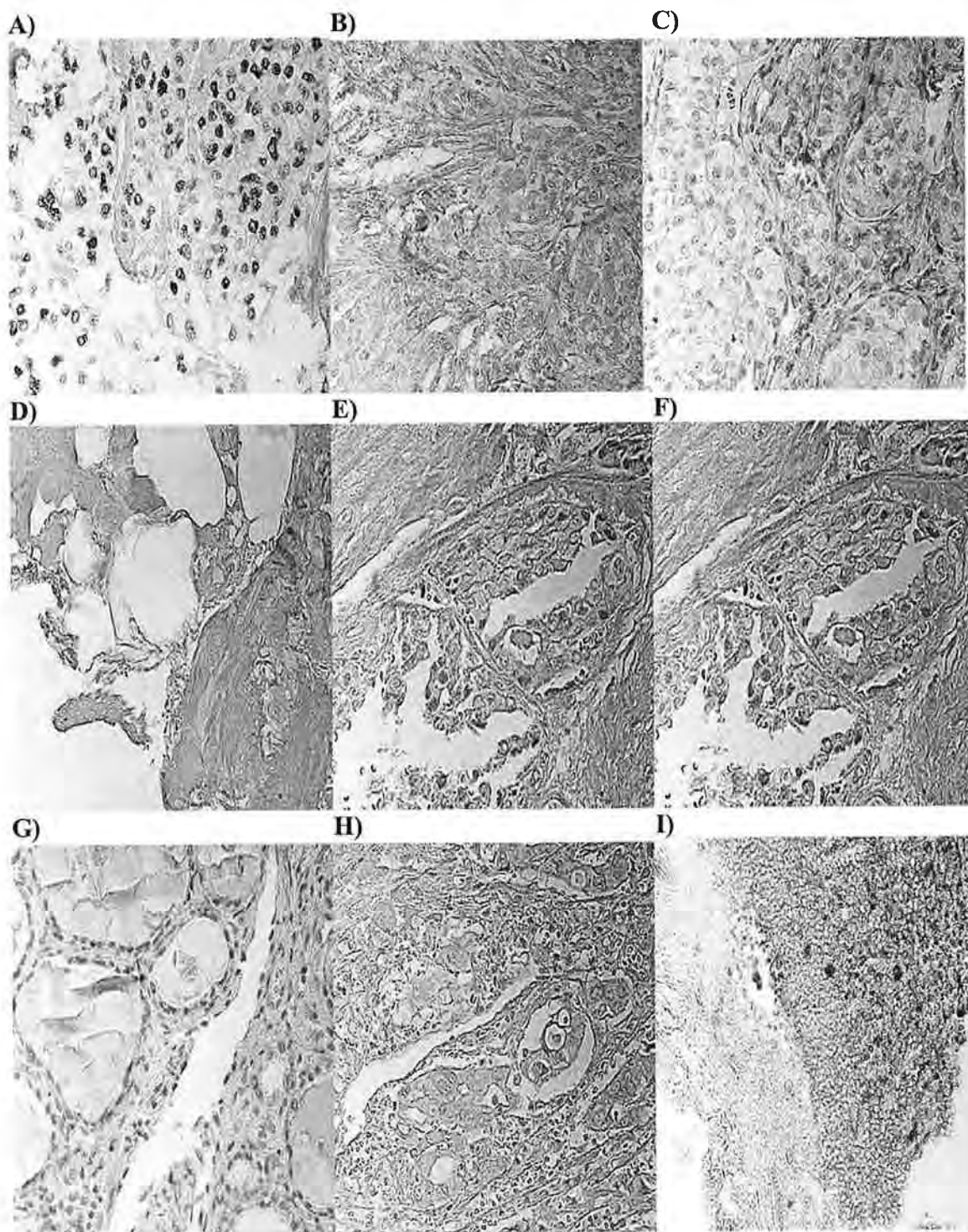


Fig 4.1 ER-1 receptor expression on human ATC.

Immunohistochemical staining of paraffin embedded tissue using a rabbit anti human ER-1 monoclonal antibody (clone SP1) demonstrated that the significant majority of ATC are negative for ER-1 receptor. Human breast cancer tissue was used for the positive control (A). One sample from n=8 assessed stained positive for ER-1 (I).

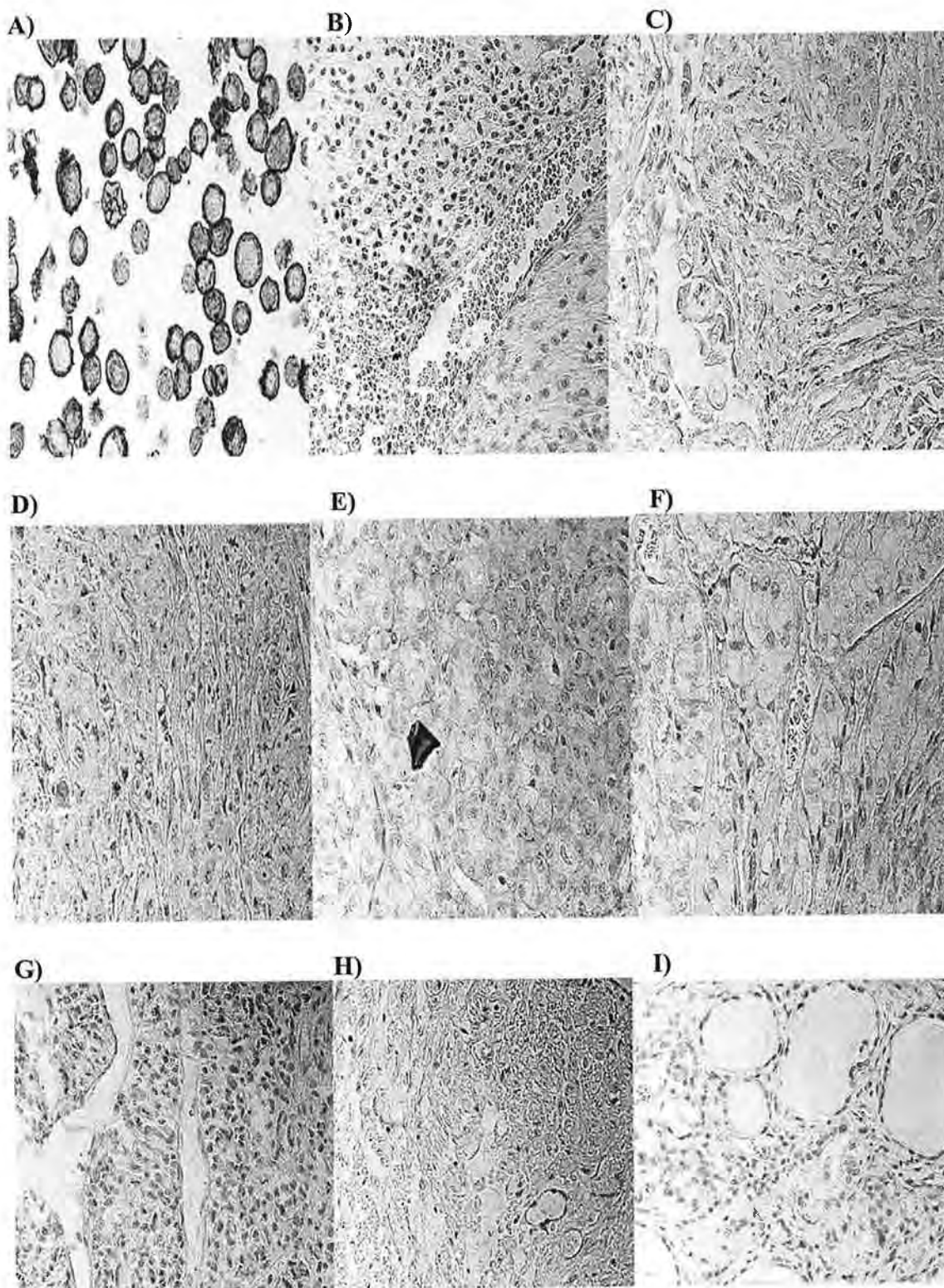


Fig 4.2(a) Her-2 receptor expression on human ATC.

Immunohistochemical staining of paraffin embedded tissue using the HercepTest® demonstrated that the significant majority of ATC are negative for Her-2 protein expression . Human breast cancer tissue (SK BR-3) was used for the positive control (A). One sample from n=8 assessed stained positive for Her-2 (E).

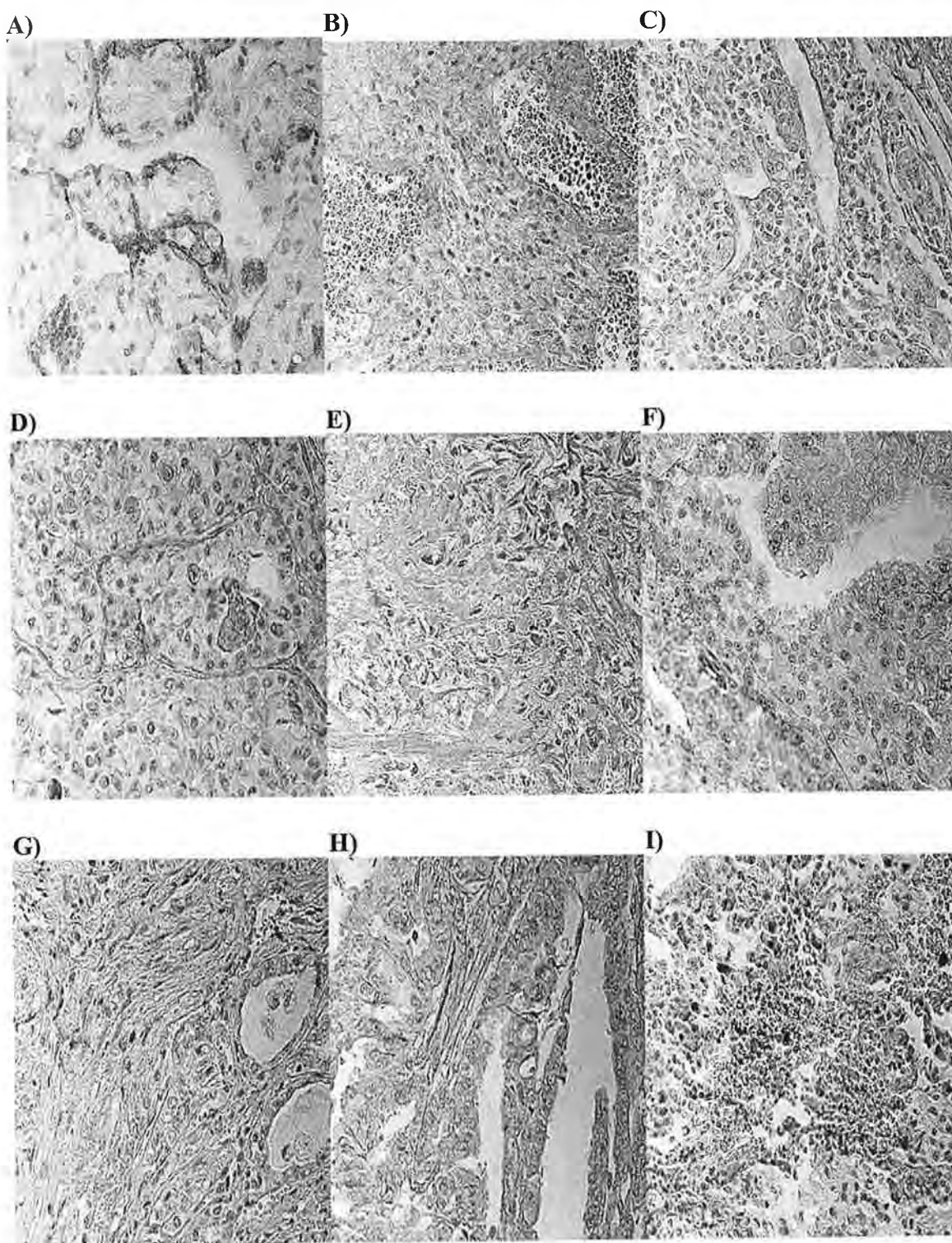


Fig 4.2(b) EGFR receptor expression on human ATC.
Immunohistochemical staining of paraffin embedded tissue using a rabbit anti human EGRF monoclonal antibody (clone SP1). Human placenta tissue was used for the positive control (A). One human sample from n=8 assessed stained positive for EGFR.

Discussion

Estrogen Receptor

Within these samples we observed three positive samples. 6324-3 was positive for the Estrogen Receptor. At the time this work was carried out ER-(was the clinically relevant receptor in relation to medicating tamoxifen's effects on cell function. Furthermore no reliable ER-(antibody was available for staining paraffin embedded tissue. 8608 was positive for HER and 6324-3 was also positive for EGFR.

(Egawa C et al 2001) performed a quantitative analysis of estrogen receptor-alpha (ER-alpha) and -beta (ER-beta) mRNA expression in normal thyroid and tumor tissues. They concluded that a downregulation of ER-alpha mRNA in follicular and anaplastic carcinomas seems to suggest that estrogens are unlikely to play an important role in the carcinogenesis and progression of these carcinomas.

However they also observed a significant decrease in ER-beta to ER-alpha mRNA ratios in follicular adenomas suggests a possible involvement of estrogens in the pathogenesis of this disease since the same phenomenon has been reported on estrogen-dependent breast cancers. Without an exact biochemical route linked in the post malignant dedifferentiation pathway this remains an interesting association especially as one of our samples was ER positive.

Takeichi et al 1991 also examined the relationship between the histological grade of dedifferentiation of thyroid cancer and estrogen receptors (ER) immunohistochemically. Six cases of poorly differentiated papillary cancer, 5 (83.3%) had 1-19 Estrogen Receptor –Immuno reactive cells per visual field. Estrogen Receptor –Immuno Reactive cells were negative in 5 out of 6 cases (83.3%) of Anaplastic thyroid cancers. Thus, the number of

Estrogen Receptor –Immuno Reactive cells tended to decrease with the degree of atypism of thyroid cancer. We would agree from our results the Estrogen Receptor does not appear to play an important role in this disease.

Epidermal Growth Factor Receptor

Ensinger C *et al* (2004) assayed twenty-five ATCs, including 3 ATCs with poorly differentiated thyroid carcinoma (PDTC) parts, by immunohistochemistry, with a mouse monoclonal antibody directed against EGFR (EGFR pharmDX kit). The tumors revealed primarily a distinct membranous staining pattern, and in several tumor cells an additional cytoplasmic reactivity could be observed. The anaplastic carcinomas presented with 5 of 25 (20%) without EGFR reaction, 10 of 25 (40%) with reactivity, and 10 of 25 (40%) with overexpression of the receptor.

They concluded that for at least one-third of all ATC tumours, EGFR seems to be a promising agent for the targeted molecular therapy of these extraordinarily aggressive tumors.

In our study we found two/eight of our samples expressed either EGFR or Her-2. Our results of 25% expression therefore agree with Ensinger et al however we did expect a higher figure. This may be related to a lack of antigenicity within the samples.

EGFR expression has implications for the Tamoxifen chapter as overexpression of Her-2/neu is associated with Tamoxifen resistance, however this appears to only occur in the presence of the Estrogen Receptor.

In vitro studies have showed that EGFR tyrosine kinase inhibitors (TKIs) greatly inhibited cellular growth and induce apoptosis in ATC cell lines. Of note somatic mutations in the tyrosine kinase domain or an increased gene copy number are associated with increased sensitivity to TKIs in non-small cell lung cancer.

Lee et al in 2006 investigated the EGFR gene status and protein expression by direct DNA sequencing of the hot-spot regions in exons 18, 19 and 21, fluorescence in situ hybridization (FISH), and immunohistochemistry in tumor tissues from 23 patients with ATC.

They did not find on mutational analysis and FISH, mutation in the hot-spots nor was gene amplification observed. Yet, high polysomy was identified in 14 (60.9%) of 23 patients with ATC. All cases with Immunohistochemistry positivity (n=6) had high polysomy, whereas 8 (47.1%) out of the 17 cases with Immunohistochemistry negativity had high polysomy (P=0.048).

This may indicate that tyrosine kinase inhibitors (TKI) may not have the impact expected however despite the low incidence of somatic EGFR gene mutation and amplification in the study samples, when considering that high polysomy was often identified by FISH as well as the current lack of therapeutic options, EGFR TKIs are worth investigating for treating the patients with ATC.

ATC is a locally aggressive tumour with high rates of distant metastases. Conventional therapies give survival in terms of 4- 12 months. New treatment options are required and targetted therapies may ultimately prove to be the desired path.

We found a definite need for a European BioBank with Anaplastic cell lines on offer. During the course of this work we contacted Dr Sugawara, one of the leading ATC

Researchers in Japan. He sent our laboratory, on two occasions, cell lines he engineered from Japan. Unfortunately the cells died in transit. We appreciate the numbers are low but this reflects the difficulty establishing cell lines and tissue from patients for research purposes. We still believe these samples offer a good insight into ATC biology.

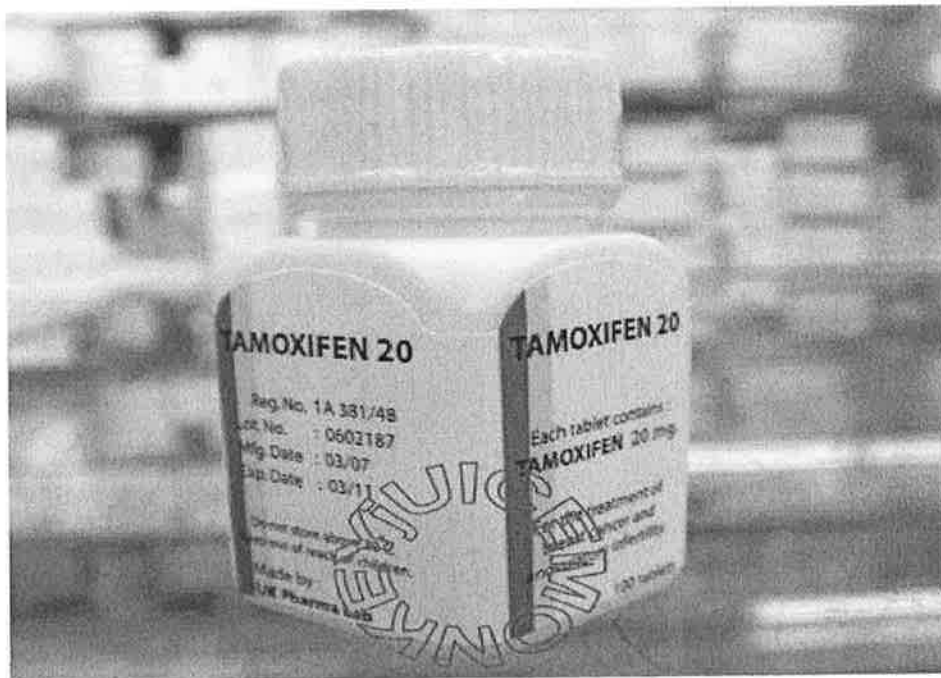
Our immunohistochemical results lead us to begin work on two novel chemotherapeutic agents. 17-(allylamino)-17-demethoxygeldanamycin (17 AAG), a derivative of the ansamycin antibiotic Geldanamycin and to investigate the non estrogenic properties of the most successful drug in the history of cancer therapy, Tamoxifen.

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Chapter Five

Tamoxifen as a potential chemotherapeutic agent

For Anaplastic Thyroid Cancer



The Most Successful Chemotherapeutic Drug in Oncology

5.1 Introduction

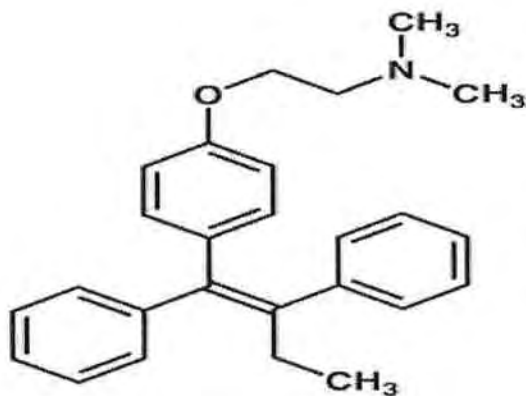
Anaplastic Thyroid Cancer (ATC) is a highly aggressive neoplasm with poor response to current therapy and mean survival of 3-7 months. This is a rare neoplasm representing <5% of all thyroid cancers however there is evidence that the incidence of this disease is growing. This cancer has a very low cure rate with the very best treatments allowing only 10 % of patients to be alive 3 years after diagnosis. ATC often arises within a more differentiated thyroid cancer or within a long standing goitre. Like papillary cancer, anaplastic thyroid cancer may arise many years (>20) following radiation exposure. Cervical metastasis (spread of the cancer to lymph nodes in the neck) are present in the vast majority (over 90%) of cases at the time of diagnosis. The presence of lymph node metastasis in these cervical areas causes a higher recurrence rate and is predictive of a high mortality rate. The majority of ATC patients develop metastases during their illness and 20- 60% of ATC patients have metastases on presentation. (O'Neill JP., *et al.* 2005) The metastatic potential and biological aggression of this tumour variant highlights the essential need for *effective systemic chemotherapy*.

Doxorubicin, cisplatin and paxlitaxel to date offer poor chemotherapeutic response. A National Cancer Institute trial (ICC 2302) of induction doxorubicin/cisplatin followed by combretastatin A4 Phosphate and RT for newly diagnosed regionally advanced ATC is accruing. Combretastatin A4 phosphate (CA4P) represents the lead compound in a group of novel tubulin depolymerising agents being developed as vascular targeting agents.

Preclinical studies have shown that CA4P induces blood flow reductions and subsequent tumour cell death in a variety of preclinical models.

Since its introduction in 1971, tamoxifen has proved to be effective – first in palliative treatment of advanced breast cancer and later, as an adjunct to surgery in early tumours. More recently, the activity of tamoxifen in chemoprevention of breast cancer has also been demonstrated, although the absolute value of such treatment remains a matter of controversy.

The anti-tumour effects of tamoxifen are not entirely understood but is believed to result from competitive interaction with the oestrogen receptor as a frank oestrogen, as an agonist or as an antagonist, depending on the species studied, the target organ assessed and the amount of tamoxifen used. Estrogens work along with genetic changes to promote the development and growth of breast cancers. Because estrogenic hormones act via the estrogen receptors (ERs), ER-alpha and ER-beta, and the ER is present in more than half of breast tumors, this receptor has been the most widely targeted protein in breast cancer therapy. The presence of the ER in breast tumors predicts improved disease-free survival and response to selective ER modulators (SERMs), such as tamoxifen, or other forms of endocrine therapy. Suppression of ER activity by SERMs has proven to be a great benefit in the treatment of breast cancers and also in the prevention of breast cancer in women at high risk for the disease. (Katzenellenbogen et al, 2005)



□ *Molecular Structure of Tamoxifen*

Not all effects of tamoxifen can be explained by mediation through the oestrogen receptor as 14–30% of oestrogen-receptor-negative human breast cancer patients have been reported to respond to tamoxifen therapy.

It has been previously demonstrated that tamoxifen reduces serum levels of VEGF, which is a prime regulator of tumour angiogenesis, in cancer patients. A number of non estrogenic cancers have responded to Tamoxifen therapy (Perez et al, 2003, Fine et al 2006, Konstantinova et al 2005, Tang et al 2006) We investigated this response. The aim of this chapter was to determine if tamoxifen could act directly on ATC cells.

5.2 Methods

CAL-62 and BHT101 ATC cell lines were first assessed for oestrogen receptor expression by immunohistochemistry as described (2.10.2). Both CAL-62 and BHT-101 cell proliferation, measured following (-estradiol 1nM treatment for 24hrs, showed no significant decrease in proliferation from untreated tumour cells as measured by the MTT assay. This dose has previously been used in our laboratory to stimulate breast cancer

cells (A Byrne 2007). This experiment would have been strengthened by the addition of a suitable positive control such as VEGF to stimulate tumour cell proliferation and ensure that the cells were capable of proliferation under these particular experimental conditions. The effects of oestrogen stimulation and tamoxifen on tumour proliferation was assessed by MMT assay. The percentage apoptosis at 24hr was determined using flow cytometry (2.31). Tumour metastatic potential was investigated with a Matrigel invasion assay with tamoxifen (5 uM/ml) and chemotactic agents VEGF (20 ng/ml) and EGF(50 ng/ml) .

5.3 Results

5.3.1 Tumour growth

5.3.1.1 Oestrogen receptor expression

Immunohistochemistry staining confirmed that both the CAL-62 and BHT101 cell lines are Oestrogen Receptor negative. (**Fig. 5.1**). β -estradiol stimulation produced no effect on cell proliferation in either cell line (**Fig. 5.2**) demonstrating that oestrogen does not act as a growth factor for these tumour cells.

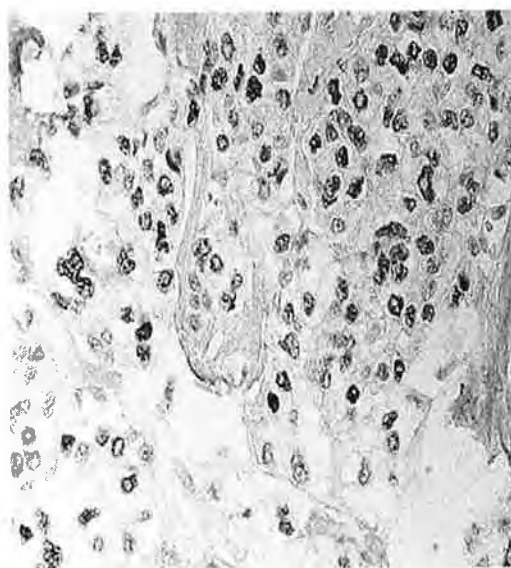
5.3.1.2 Tumour cell proliferation

Cal-62 cell proliferation, measured following tamoxifen (Hoelting et al, 1995) treatment for 24hr, showed a significant decrease from control at 1 μ M (97 % \pm 1.07), 2 μ M (93 % \pm 0.75), 5 μ M (64% \pm 1.07) and 10 μ M (23% \pm 1.07) (**Fig. 5.3a**). BHT101 proliferation was more sensitive to tamoxifen at the lower doses of 1 μ M (91% \pm 2.9) and 2 μ M (85% \pm 2.6 0.75), and less sensitive than the CAL-62 cells at the higher does of 5 μ M (81% \pm 3.36) and 10 μ M (74% \pm 3.2) (**Fig. 5.3b**)

5.3.1.3 Tumour cell apoptosis

Cal-62 apoptosis was significantly up-regulated by incubation with tamoxifen for 24hr
Control ($4.7\% \pm 0.46$) 1 μM ($11.2\% \pm 2.39$), 2 μM ($26.4\% \pm 1.4$), 5 μM ($24.8\% \pm 2.19$) and
10 μM ($22.9\% \pm 6.89$) (**Fig. 5.4a**). BHT101 apoptosis was also significantly increased by
tamoxifen ($6.6\% \pm 0.33$) 1 μM ($8.5\% \pm 1.27$), 2 μM ($18\% \pm 3.5$), 5 μM ($25.7\% \pm 1.85$) and
10 μM ($28.3\% \pm 1.33$) (**Fig. 5.4b**)

(A)



(B)

(C)

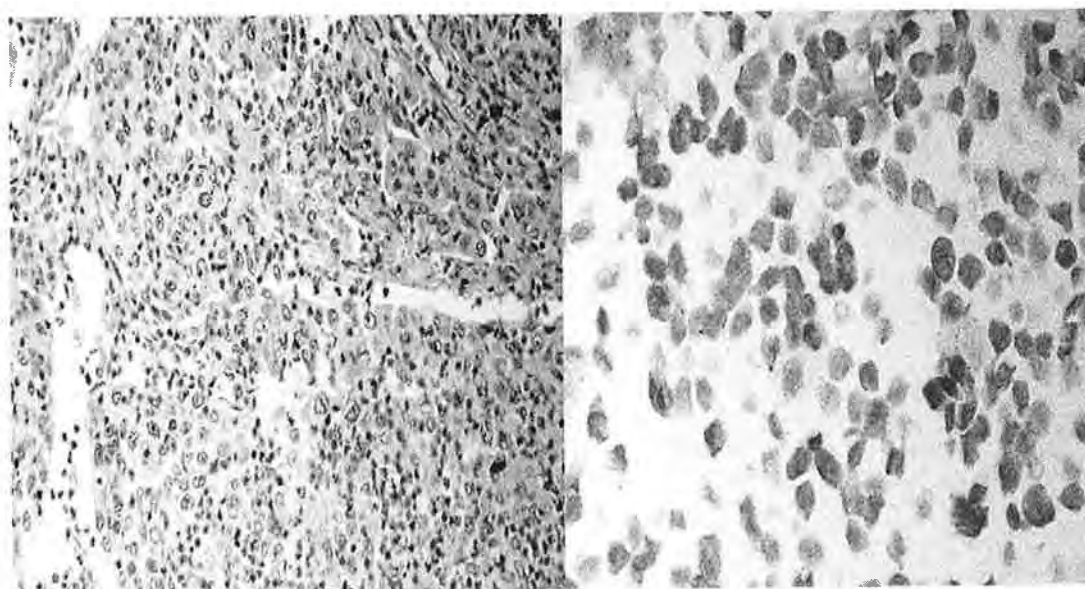


Fig 5.1 Oestrogen Receptor Expression

These photomicrographs (x 40) show immunohistochemical staining for oestrogen receptor using a rabbit monoclonal antibody (1:50 dilution). Breast Tissue used as a positive control (A) clearly demonstrates positive brown staining. CAL-62 (B) and BHT101 (C) cell lines are Oestrogen Receptor negative as seen by the absence of brown DAB staining.

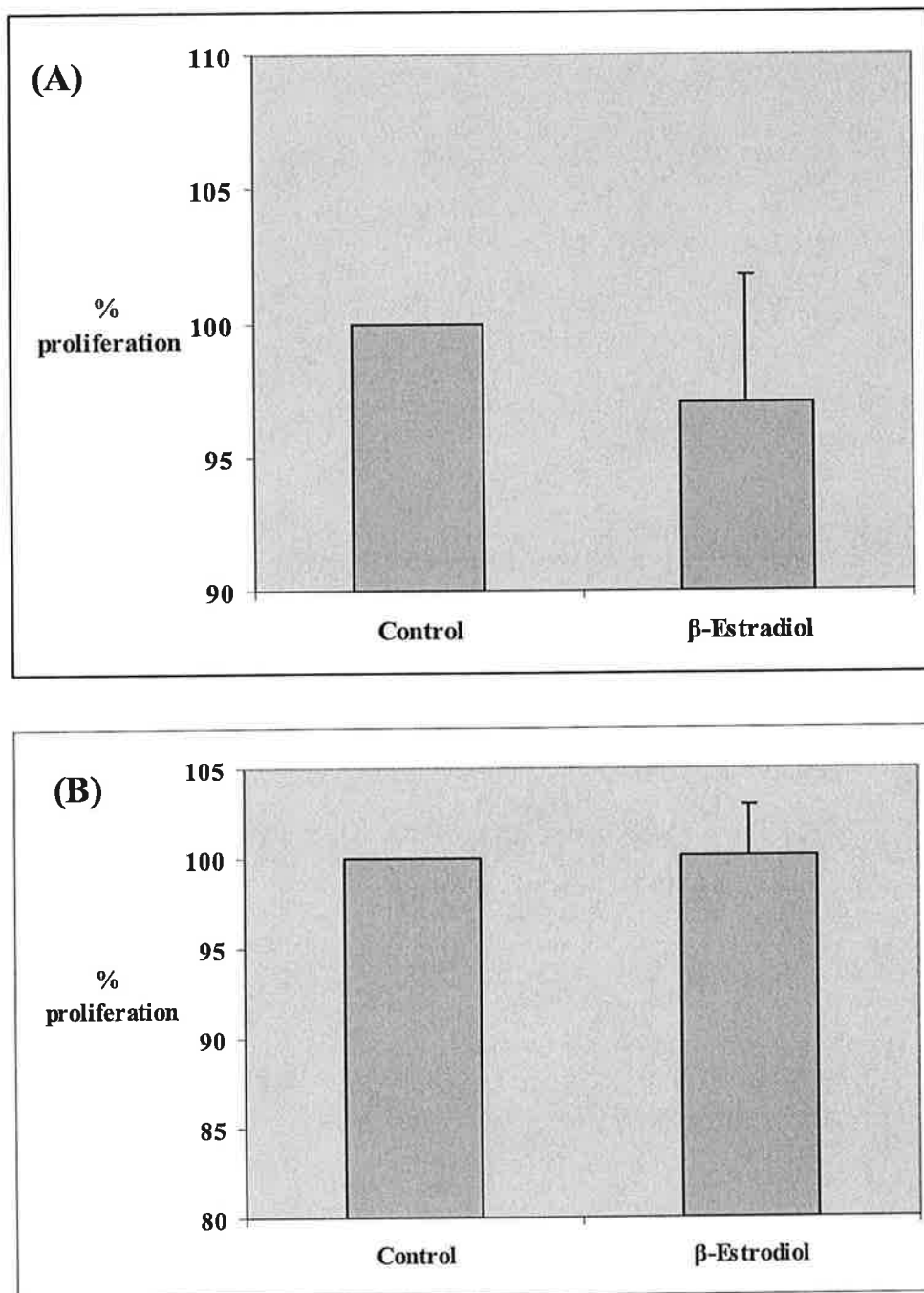


Fig 5.2 Tumour cell proliferation in response to β -estradiol

(A) CAL-62, (B) BHT 101 Cells were incubated in β -estradiol 10nmol/L (Manole et al 2001) for 24hr and proliferation measured by spectrophotometer using the MTT assay. Samples were assayed in triplicate, n= 3 individual experiments. Data is recorded as a percentage difference from control and expressed as mean \pm SEM. No significant difference from control. (p=0.62 (A) p= 0.98 (B) t-test).

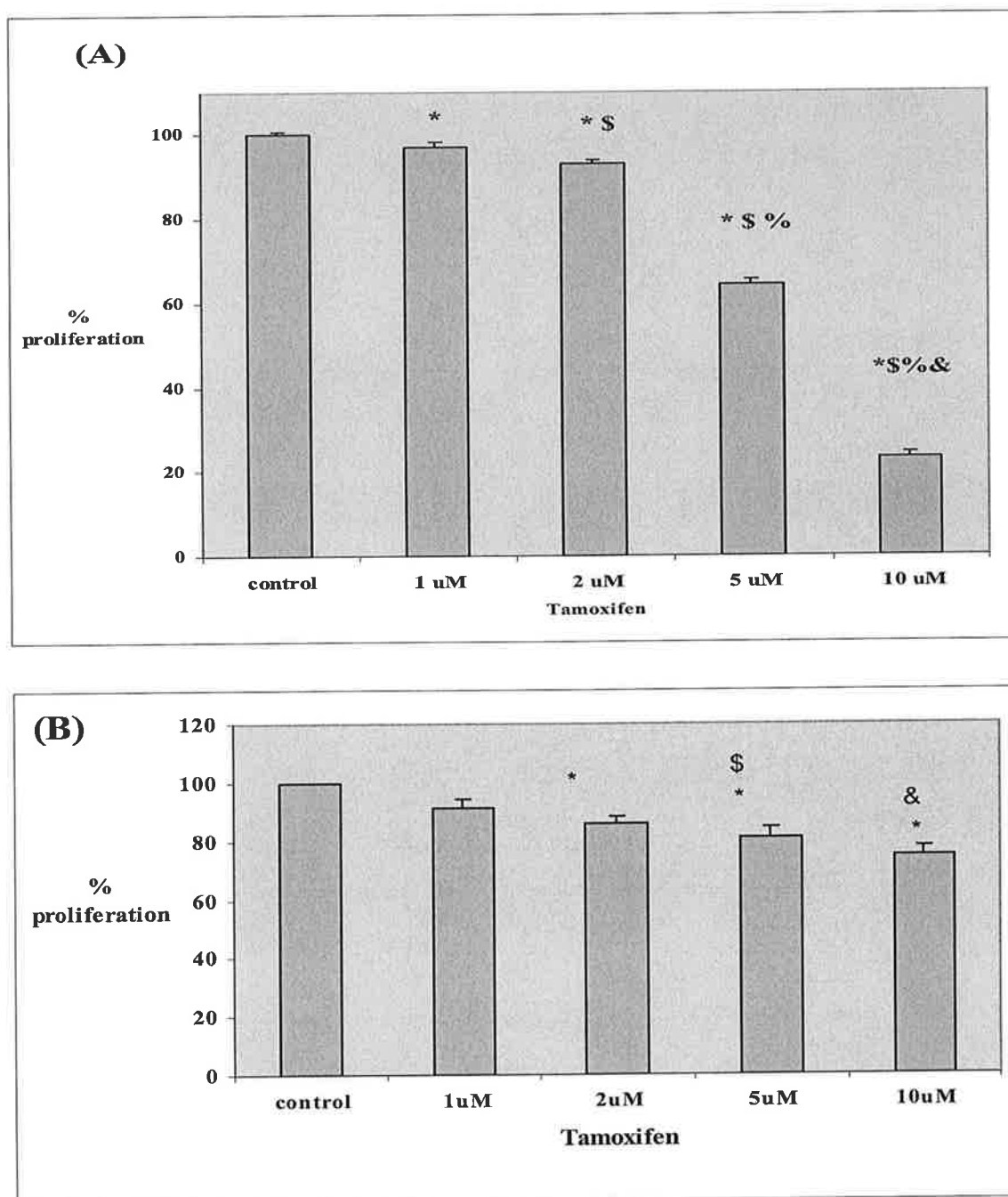


Fig 5.3 Tamoxifen retards tumour cell proliferation

CAL-62 (A) and BHT 101 (B) cells were incubated in tamoxifen for 24hr and proliferation measured by spectrophotometer using the MTT assay. Samples were assayed in triplicate, n= 5 individual experiments. Data is recorded as a percentage difference from control and expressed as mean \pm SEM. * denotes a significant difference from control. \$ denotes a significant difference from 2 μ M tamoxifen treated group & denotes a significant difference from 5 μ M tamoxifen. p<0.05, Anova Scheffe post hoc analysis

□

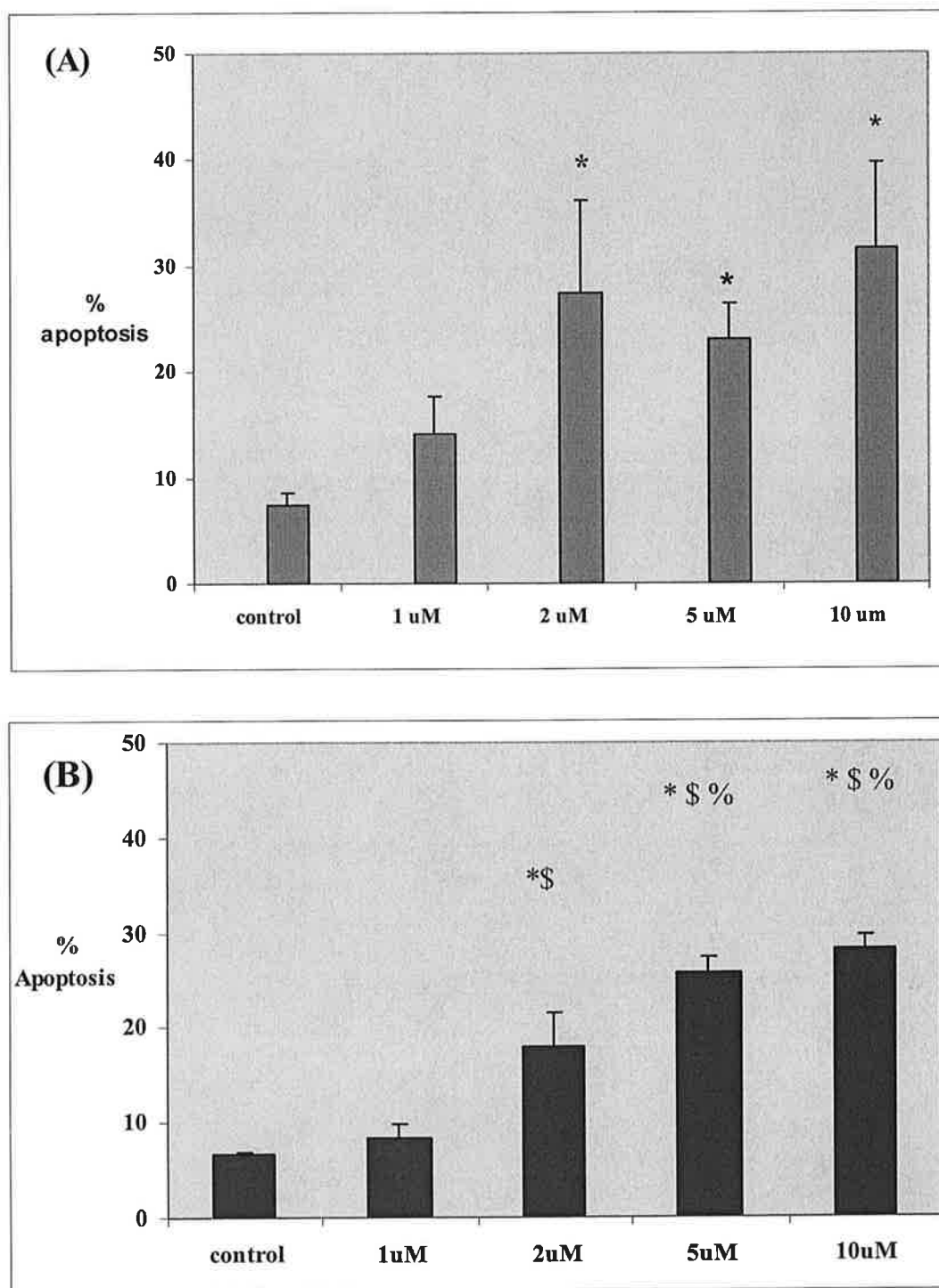


Fig 5.4 Tamoxifen induces tumour cell death

CAL-62 (A) and BHT 101 (B) apoptotic cell death was assessed following incubation in tamoxifen for 24hr by measuring Annexin V binding. Apoptosis is measured as a percentage of 10,000 cells acquired and expressed as mean + SEM. (n=6 individual experiments. * denotes a significant difference from control. \$ denotes a significant difference from 1 μ M tamoxifen treated group. % denotes a significant difference from 2 μ M tamoxifen. $p < 0.05$, Anova Scheffe post hoc analysis .

5.3.2 Metastatic potential

5.3.2.1 Vascular Endothelial Growth Factor

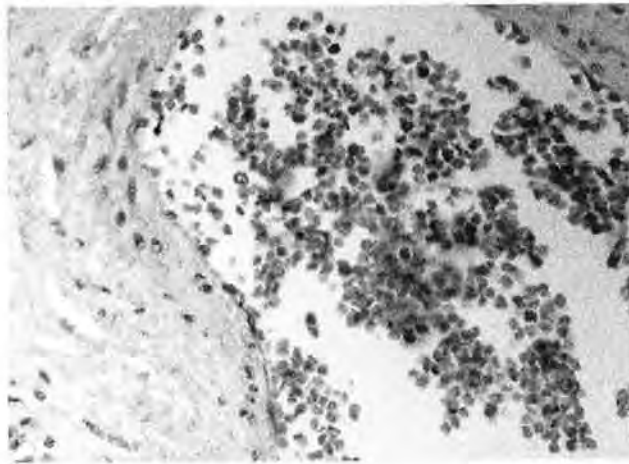
VEGF plays a paracrine and autocrine role as a growth factor for tumour cells and is considered a crucial angiogenic cytokine driving the metastatic process in a variety of malignancies. Immunohistochemical staining clearly demonstrates the production of VEGF by both cell lines (Fig 5.5)

Cell surface VEGF receptors FLT-1 and FLK-1 were assessed by western blot. Both cell lines express the FLT-1 and FLK-1 VEGF receptors, with Cal-2 demonstrating a more dense cell surface distribution of FLT-1 compared to BTH101 (Fig 5.6)

5.3.2.2 Tumour cell migration.

Metastatic potential was examined following stimulation with VEGF and tamoxifen treatment. Tamoxifen significantly reduced cell migration at 2 μ M and at 5 μ M. VEGF significantly up-regulated tumour cell migration and tamoxifen at 5 μ M prevented this VEGF mediated enhanced tumour cell migration. (Fig5.7).

(A)



(B)

(C)

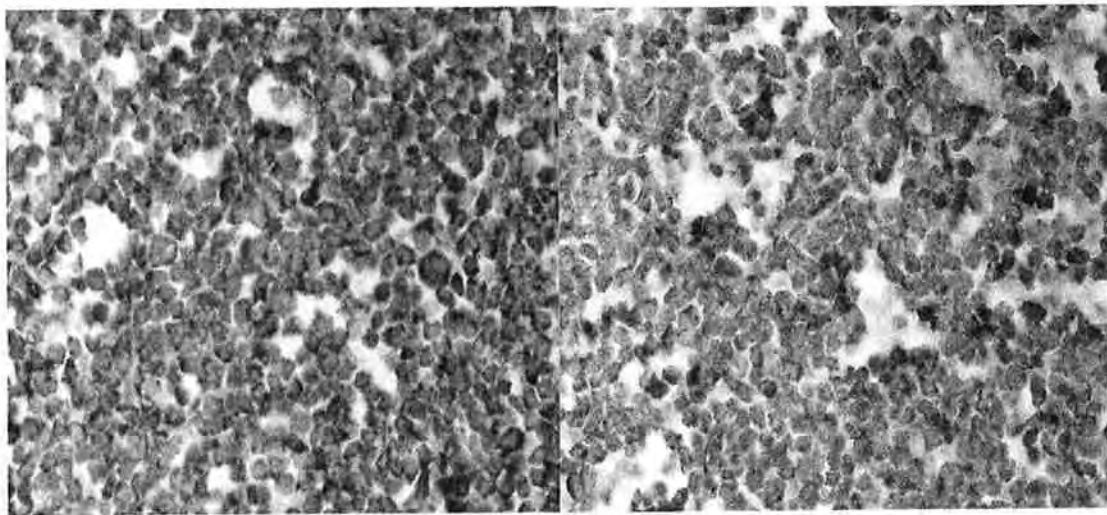


Fig 5.5 Cytosolic production of VEGF

Immunohistochemical staining for the presence of VEGF was carried out using breast tissue as a positive control and cells alone without the addition of the antibody as a negative control to ensure no nonspecific staining was occurring. These photomicrographs (x 40) show immunohistochemical staining for the presence of VEGF using a polyclonal antibody (1:100 dilution). Breast Tissue used as a positive control (A) clearly demonstrates positive brown staining. CAL-62 (B) and BHT101 (C) cell lines

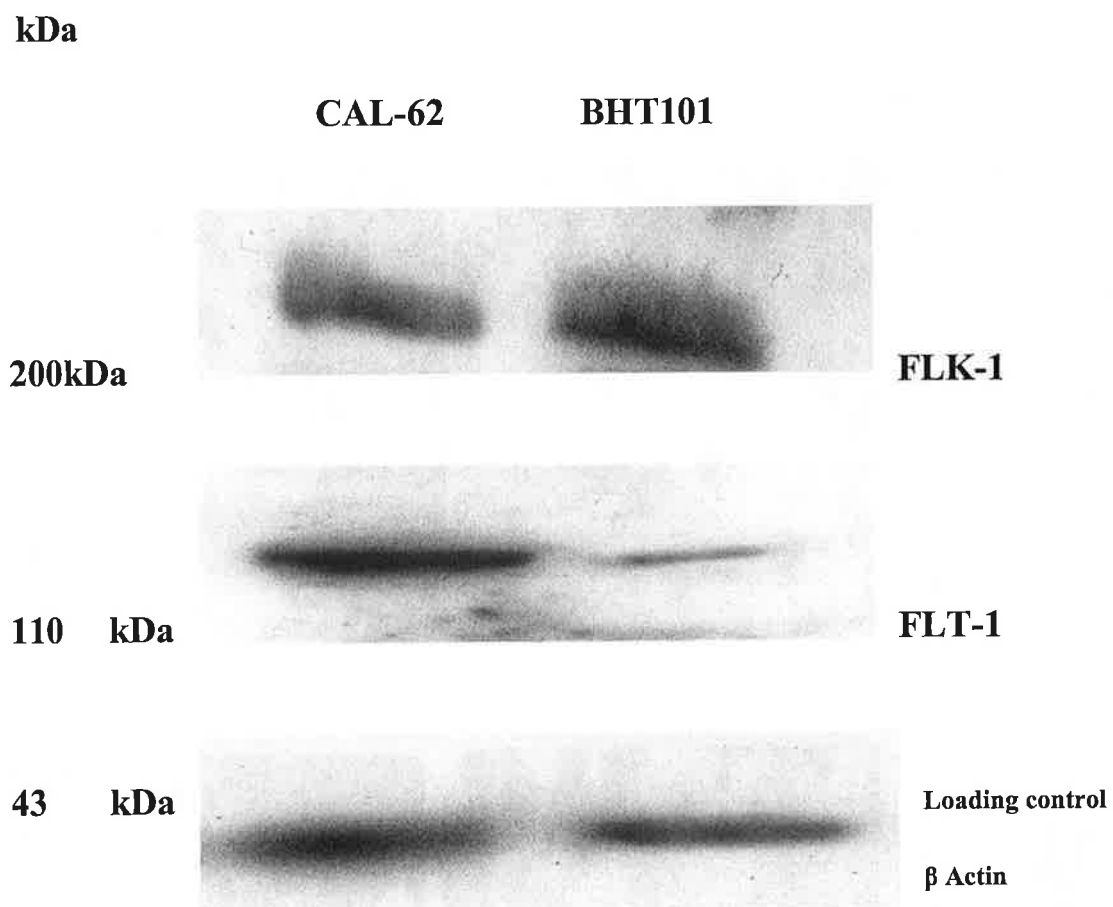


Fig 5.6 VEGF Receptor expression

VEGF receptor expression was assessed in tumour cell lysates but western blotting. FLT-1 receptor is expressed by both CAL-62 and BHT101 cells. FLK-1 is also expressed by both tumour cells. β Actin staining ensures equal protein loading.

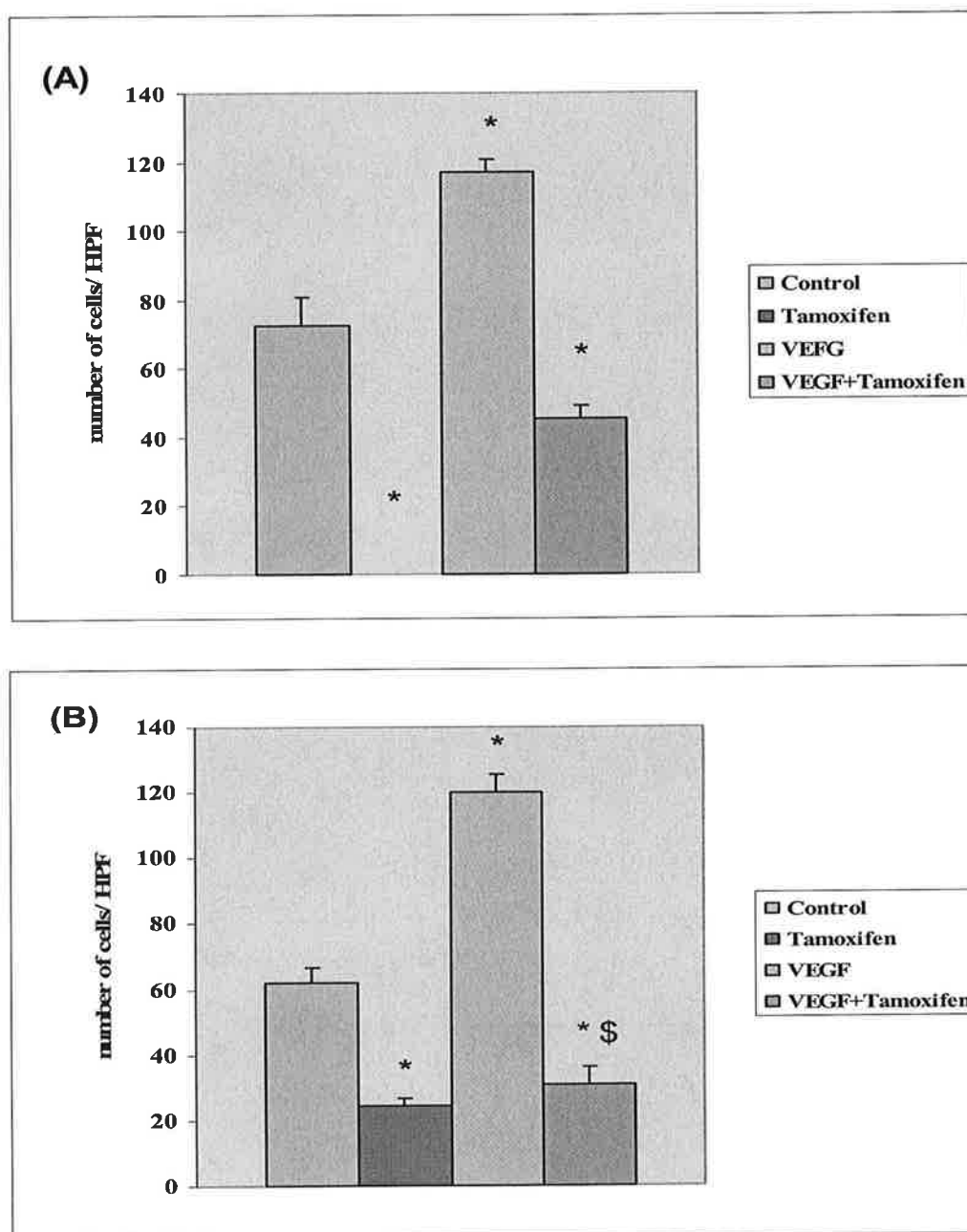


Fig 5.7 c Metastatic Potential

Tumour cell migration as assessed by transwell assay is graphically demonstrated. The results obtained using this boyden chamber assay shows a strong correlation between the ability of tumor cells to invade in vitro and their invasive behavior in vivo, which validates this assay as a measure of invasive potential. (Shaw et al 2005). Tamoxifen at 5 μ M significantly retard tumour cell migration compared to control in both Cal-62 (A) and BHT 101 (B) cells. VEGF (20ng/ml) significantly up-regulates tumour cell migration and this stimulation is prevented by tamoxifen at 5 μ M in both cell lines. Data is recorded as number of cells migrated per high power field (HPF) and expressed as mean \pm SEM. * denotes a significant difference from control. \$ denotes a significant difference from VEGF treated group. $p < 0.05$, LSD post hoc analysis. $n=3$ independent experiment.

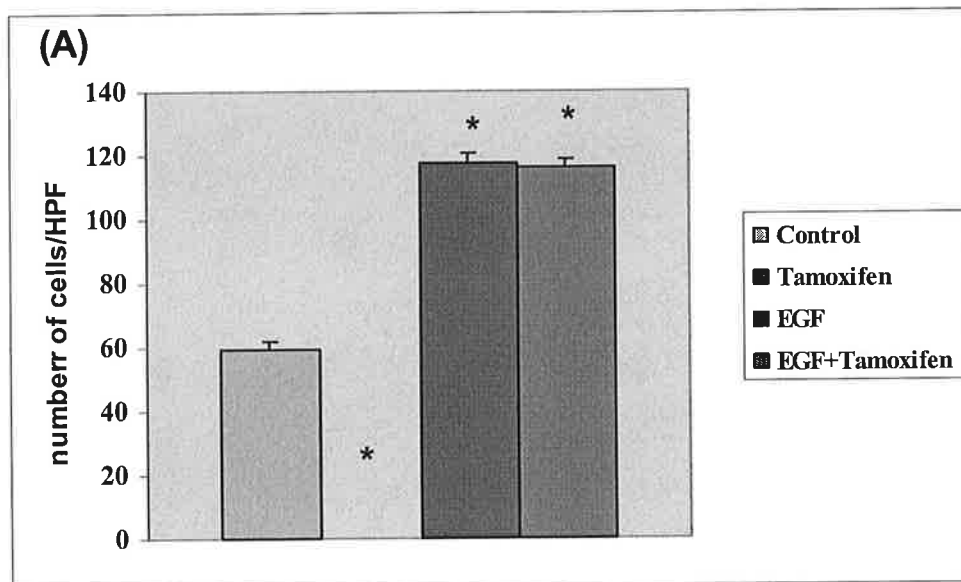


Fig 5.7 d Metastatic Potential

Tumour cell migration as assessed by transwell assay is graphically demonstrated. . Tamoxifen at 5 μ M (C) significantly retard tumour cell migration compared to control in both Cal-62 (A). EGF (50 ng/ml) significantly up-regulates tumour cell migration and this stimulation is not prevented by tamoxifen at 5 μ M in either cell lines. Data is recorded as number of cells migrated per high power field (HPF) and expressed as mean \pm SEM. * denotes a significant difference from control. \$ denotes a significant difference from VEGF treated group. $p < 0.05$, LSD post hoc analysis. $n = 3$ independent experiment. * $P < 0.000$ Vs Anova LSD post Control

5.4 Discussion

The CAL-62 and BHT-101 cell lines are highly malignant ATC cell lines. This work demonstrates that both cell lines express VEGF Receptors FLT-1 and FLK-1, produce VEGF and are estrogen receptor negative.

Tamoxifen at pharmacological dosage significantly decreases proliferation, accelerates the induction of apoptosis and downregulates the metastatic and invasive action of VEGF in both cell lines studied. Tamoxifen at micromolar concentrations is known to have profound inhibitory effects on the growth of oestrogen-receptor-negative cancer cell lines such as rat Nb₂ lymphoma human BT₂₀ breast carcinoma and human A549 lung adenocarcinoma (Jun CAI and Chee Wee LEE, 1996). Tamoxifen exhibits a wide range of biological effects that may account for its activity in ER-negative tumours inhibition of calmodulin, stimulation of transforming growth factor β secretion, induction of apoptosis, interaction with P-glycoprotein, inhibition of protein kinase and phospholipase C, stimulation of phosphoinositide kinase activity, influence on the expression of insulin-like growth factor-1 and insulin-like growth factor binding protein-1, and anti-angiogenic effect (Senkus-Konefka E. et al 2003).

Furthermore tamoxifen also acts as a membrane fluidity modulator and this is thought to be the basis of its anti-oxidant, anti-cancer, anti-viral, anti-multidrug-resistance and cardioprotective actions. In addition, muscarinic receptor, histamine-like receptors, and P-glycoprotein, are all affected by tamoxifen.

A distinct binding site for the triphenylethylene antiestrogens such as tamoxifen has been identified. These antiestrogen binding sites (AEBS) are distinct from the ER both in ligand specificity and in subcellular localization (Jordan VC. and Murphy CS. 1990; Katzenellenbogen BS. et al, 1985; Sudo K. et al, 1983).

The AEBS are widely distributed in various tissues such as cytosolic extracts of rat liver, guinea pig uterus, chick oviduct, human breast cancer MCF-7 cell line, and various other human tissues including thyroid tissue (Sutherland RL. et al , 1980; Kon OL. 1983;) . The AEBS is a hetero-oligomeric complex consisting of 3β -hydroxysterol- Δ^8 - Δ^7 -isomerase and 3β -hydroxysterol- Δ^7 -reductase. It is likely that tamoxifen is working through AEBS in our experiments.

This work demonstrates that tamoxifen exhibits potent chemotherapeutic potential treating anaplastic thyroid cancer cells at therapeutic dose between 2 and $5\mu\text{M}$. CAL-62 and BHT 101 are highly malignant anaplastic thyroid cancer cell lines. They express VEGF receptors FLT-1 and FLK-1, produce VEGF and are estrogen receptor negative. Our immunohistochemical staining for the presence of VEGF was carried out using breast tissue as a positive control and cells alone without the addition of the antibody as a negative control to ensure no nonspecific staining was occurring.

Both Cal-62 and BHT101 cell proliferation, measured following β -estradiol treatment for 24hr, showed no significant decrease in proliferation from untreated tumour cells as measured by the MTT assay. The dose employed for the stimulation with β Estradiol was 10nmol/L yielding a physiological dose of estradiol (Manole et al, 2001). This experiment would have been strengthened by the addition of a suitable positive control

such as VEGF to stimulate tumour cells proliferation and ensure that the cells were capable of proliferation under these particular experimental conditions.

Both CAL-62 and BHT-101 apoptosis was significantly up-regulated compared to control non stimulated cells in a dose dependent manner. This experiment would have been strengthened by the addition of a suitable positive control with these experiments such as TNF to stimulate tumour cells apoptosis and thus providing a comparison between tamoxifen and a known inducer of apoptosis. Until recently, most of the emphasis in cancer research has been on the pathogenesis of primary tumours. Most cancer deaths, however, are the result of metastatic disease. Researchers are only just beginning to understand how cancer cells escape from their original location, travel throughout the body and select a new site at which to form new tumours, and metastasis research is one of the most rapidly developing areas of cancer biology. The Matrigel invasion assays using the Boyden Chamber reveal the ability Tamoxifen has in attenuating the metastatic potential of this disease.

Clinical trials have confirmed the efficacy of Tamoxifen in combination with conventional chemotherapy in a variety of non breast cancers. Perez et al, 2003 conducted a phase I study of high-dose oral tamoxifen in combination with intravenous cisplatin. They determined the tolerability the daily tamoxifen dose required to achieve serum levels equivalent to in vitro concentrations reported to enhance cisplatin cytotoxicity in preclinical models. Tamoxifen was administered to 15 advanced stage lung malignancies days one through seven at escalating daily doses of 160 mg/m² (n = 5), 200 mg/m² (n = 6), and 250 mg/m² (n = 4) followed by cisplatin at 100 mg/m² on day eight. Serum concentrations of tamoxifen and its hydroxylated metabolite N-

antiproliferative action. Since its discovery in 1992, MRP1 has been the most studied among MRP proteins, which now count nine members. Besides the biological work, which targets structure elucidation, binding sites location, and mode of action, most efforts have been focused on finding molecules which act as MRP1 inhibitors.

The multidrug resistance-associated protein (MRP), another member of the *mdr* gene family, may be involved in anti-cancer drug resistance of this carcinoma. The MRP expression examined immunohistochemically in 8 cell lines and 73 thyroid cancer tissues; its frequency in anaplastic carcinoma (52%) was significantly higher than that in other thyroid cancer types (Sugawara et al 1994).

Tamoxifen was investigated to observe the effect on multidrug resistance (MDR) of colorectal carcinoma in vivo. The expression of *mdr1* gene corresponds to the sensitivity of colon cancer to anti-tumor drugs in vivo. Tamoxifen can reverse the MDR of colorectal carcinoma in nude mice, which is independent of the expression of the estrogen receptor (ER); however, no changes were observed in the expressive level of *mdr1* mRNA (Shen et al 2005). Given this MRP downregulation Tamoxifen may be of benefit as a sole chemotherapeutic agent or indeed as a synergistic agent to promote Cisplatin efficacy (Mc Clay et al 1993) and penetrance.

Tamoxifen exhibits potent chemotherapeutic potential treating ATC cells in vitro and replication in vivo model. We have observed Tamoxifen decreases proliferation, accelerates the induction of Apoptosis and downregulates the metastatic, invasive and anti-apoptotic action of VEGF. Based on this data, and that of the Literature, a Phase II study of high-dose tamoxifen in combination with cisplatin in patients with Anaplastic Thyroid Cancer is warranted.

Chapter Six

17 Allylamino 17 demethoxygeldanamycin as a potential chemotherapeutic agent

For Anaplastic Thyroid Cancer

6.1 Introduction

The epidermal growth factor receptor (EGFR) family including EGFR (also known as ERBB1 and Her-1), ERBB2 (Her-2), ERBB3 (Her-3) and ERBB4 (Her-4) are transmembrane receptor proteins found primarily on cells of epithelial origin and are known to be over expressed and related to poor prognosis in some cancers.

All are involved in the transmission of signals that control cell growth and differentiation (Rubin, I. and Yarden, Y, 2001). Thyroid tumours overexpress EGFRs and ligands, implicating EGFR signalling in thyroid tumorigenesis (Kato, S. *et al*; 2004; Schiff, B. A. *et al*; 2004)

Although Her-2 amplification is important in breast cancer, no expression of Her-2 has been found in anaplastic carcinomas (Gumurdula *et al* , 2003) and no activating mutations or DNA amplification of EGFRs were found in human thyroid neoplasia (Haugen, D. R. *et al* ;1996; Sugg, S. L. *et al*; 1998).

Her-2 has no clear prognostic significance however increased expression of EGFR has been shown to correlate with poor prognosis (Akslen, L. A. and Varhaug, J. E. 1995 : Chen, B. K. *et al*; 1999) . For at least one third of all anaplastic thyroid carcinomas, EGFR seems to be a promising agent for the targeted molecular therapy (Ensinger *et al*.; 2005)

The Ras-Raf-MEK-ERK (ERK) pathway plays a central role in regulating cellular growth by relaying extracellular signals from the ligand-bound cell surface tyrosine kinase receptors EGFR and Her-2 to the nucleus via a cascade of specific phosphorylation events beginning with activation of the Ras oncogene family (Eccles SA, *et al* ; 2004).

factors such as VEGF, avoidance of apoptosis by BAD inactivation and caspase inhibition, and resistance to radiation and chemotherapy by induction of the multidrug resistance gene (*MDR-1*) (Kolch W., et al; 2002 : Giuliani N., et al 2004).

A novel anti-Raf therapeutic approach involves destabilizing the Raf kinase protein with 17-allylamino-17-demethoxygeldanamycin (17-AAG), the benzoquinone ansamycin antibiotic. Heat-shock protein 90 (Hsp90) is a molecular chaperone involved in three-dimensional folding, intracellular translocation and degradation of multiple key regulatory proteins. Accumulated evidence has indicated an important role of Hsp90 in several signal transduction pathways that are deregulated in carcinogenesis. 17-AAG, a selective inhibitor of Hsp90, is currently under clinical investigation in advanced malignancies in which Hsp90 client proteins are implicated.

17-AAG specifically binds Hsp90 at its ATP-binding site, causing disruption of stabilization, trafficking and folding of related client proteins such as HER-2 (Andreyev HJ, et al ; 998 : Nelson HH, et al ; 1999), Raf-1 (Chang F, et al ; 2003), Akt (Hagemann C, et al; 1999) and mutant p53.

17 AAG has emerged as a candidate for molecular-targeted therapies for neuro-degenerative diseases (Waza et al 2006), might be of therapeutic value in Hodgkin's Lymphoma (Georgakis et al 2006), has shown excellent responses in some melanoma models (Burger et al 2004) and was shown to arrest growth of tumour cells and apoptosis and degrade client proteins including c-Raf-1, AKT and inhibition of AKT phosphorylation in an evaluation against Head and Neck squamous cell carcinoma lines (Yin et al, 2005). 17 AAG disrupts the Raf-1-hsp90 multimolecular complex leading to

Raf-1 destabilization and degradation via cellular proteolytic mechanisms such as the proteasome-mediated pathway. This is therefore an exciting drug with many properties. The aim of this chapter was therefore to investigate anti neoplastic properties of 17 AAG. We examined Proliferation chemiluminescence assays, Apoptosis flow cytometric assays and *in Vivo* (Boyden Chamber) replication model assays. Within the Boyden Chamber we investigated the response with EGF as the chemo-attractant. We employed the ATC cell lines CAL- 62 and BHT 101.

6.2 Methods

We used CAL-62 and BHT101 anaplastic thyroid tumour cell lines. The effects of 17 AAG on tumour proliferation were assessed by MMT assay (2.xx). The percentage apoptosis at 24hr was determined using flow cytometry (2.3.1). Tumour metastatic potential was investigated with a Matrigel invasion assay with 17AAG (5 uM/ml) and EGF (50 ng/ml). (2.11) The cells were assessed for EGFR and Her-2 receptor expression by immunohistochemistry as described (2.10.2).

6.3 Results

6.3.1 Tumour growth

6.3.1.1 EGF receptor expression

Immunohistochemistry staining confirmed that the CAL-62 cell line is EGFR positive and CAL-62 is Her-2 positive. The BHT101 cell line was not EGFR positive or Her-2 positive. Controls for EGFR were Placental tissue (fig 6.1 A). Control for Her-2 was the Her-2 positive SK-BR-3 cell line (fig 6.1 B).

6.3.1.2 Tumour cell proliferation

Cal-62 cell proliferation, measured following 17AAG treatment for 24hr, showed a significant decrease from control at 100 nM ($60\% \pm 0.69$), 1 uM ($55\% \pm 0.98$), 10 μ M ($32\% \pm 0.31$) and 100 uM ($27\% \pm 0.95$) (MTT results Fig. 6.2). BHT101 decreased from control at 100 nM ($82\% \pm 0.55$), 1 uM ($71\% \pm 1.72$), 10 uM (44.0 ± 1.33) and 100 uM (37.0 ± 0.96).

6.3.1.3 Tumour cell apoptosis

Cal-62 apoptosis was significantly up-regulated by incubation with 17 AAG for 24hr Control ($7.5\% \pm 1.3$), 100nM ($10.7\% \pm 4.8$), 1 μ M ($33.8\% \pm 16.1$) and 10 uM ($36.4\% \pm 17.2$) (Fig. 6.3a). BHT101 apoptosis was also significantly increased by 17AAG control ($10.8\% \pm 0.52$) 100nM ($5.69\% \pm 0.34$), 1 μ M ($16.1\% \pm 0.53$), 10 uM ($19.0\% \pm 1.30$) (Fig. 6.3b).

A)

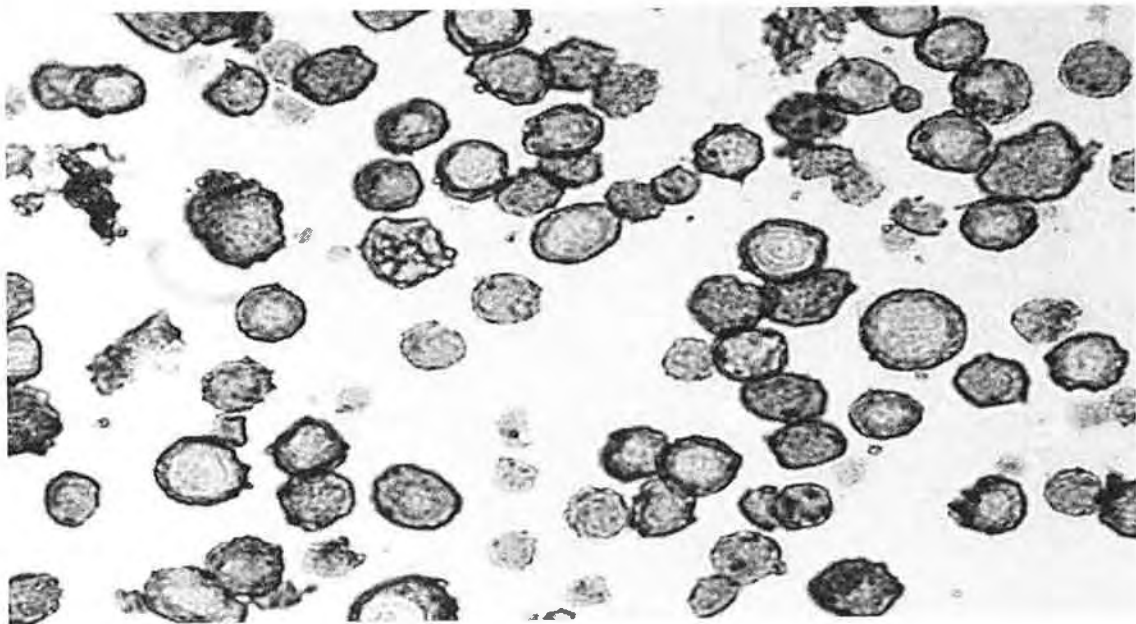
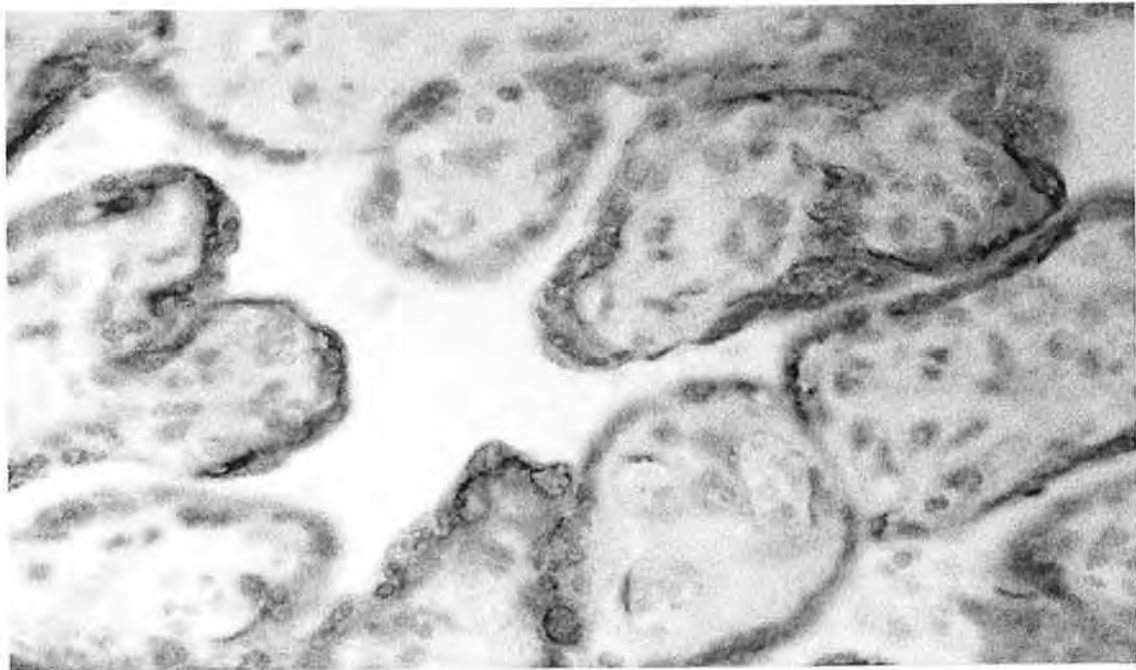


Fig 6.1 Her-2 Positive (SK-BR-3)

These photomicrographs (x 40) show immunohistochemical staining for EGFR using a rabbit monoclonal antibody (1:50 dilution). Placenta tissue used as a positive control (A) clearly demonstrates positive brown DAB staining. Negative control were cells stained with secondary antibody only to ensure no nonspecific binding.

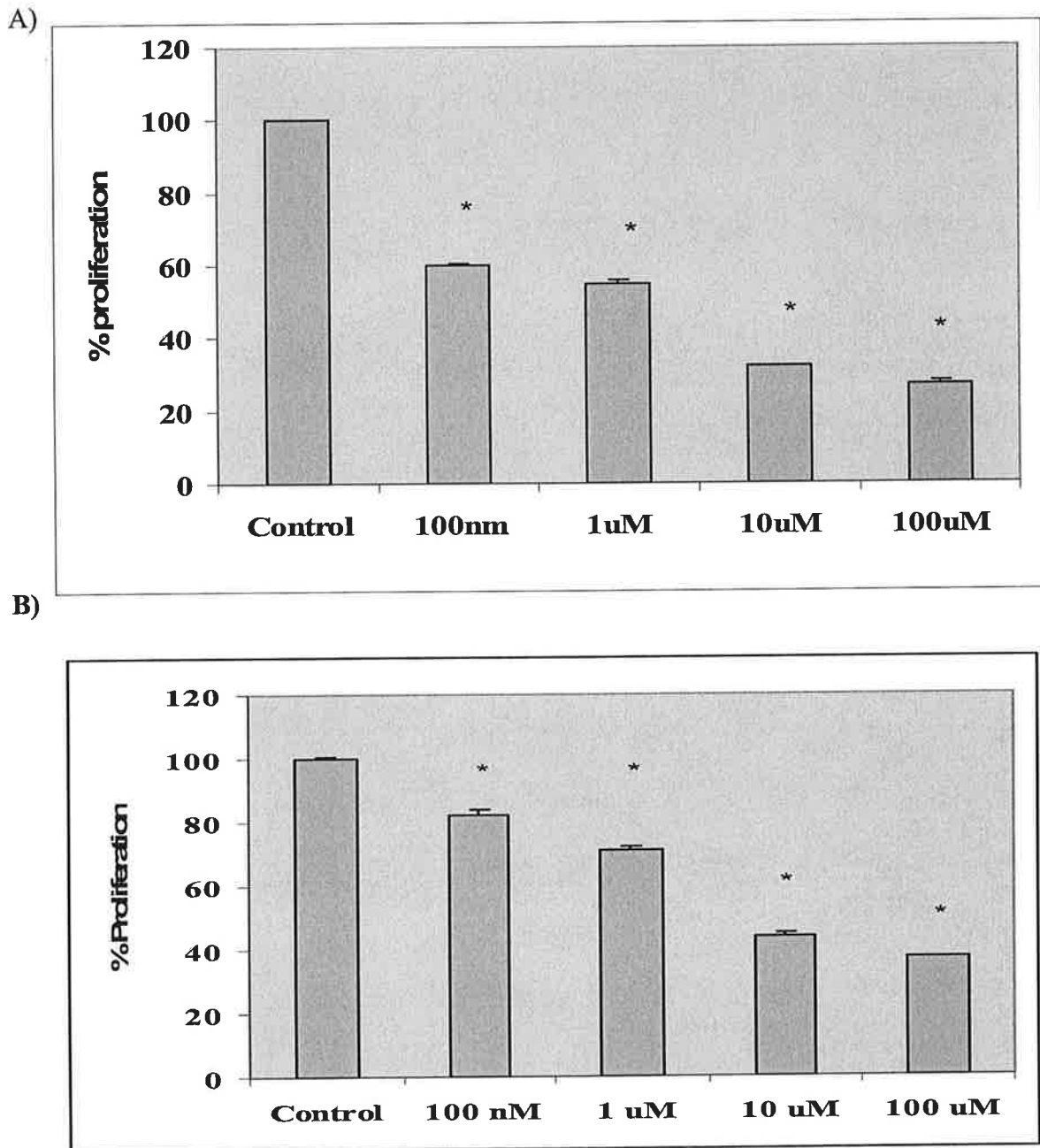
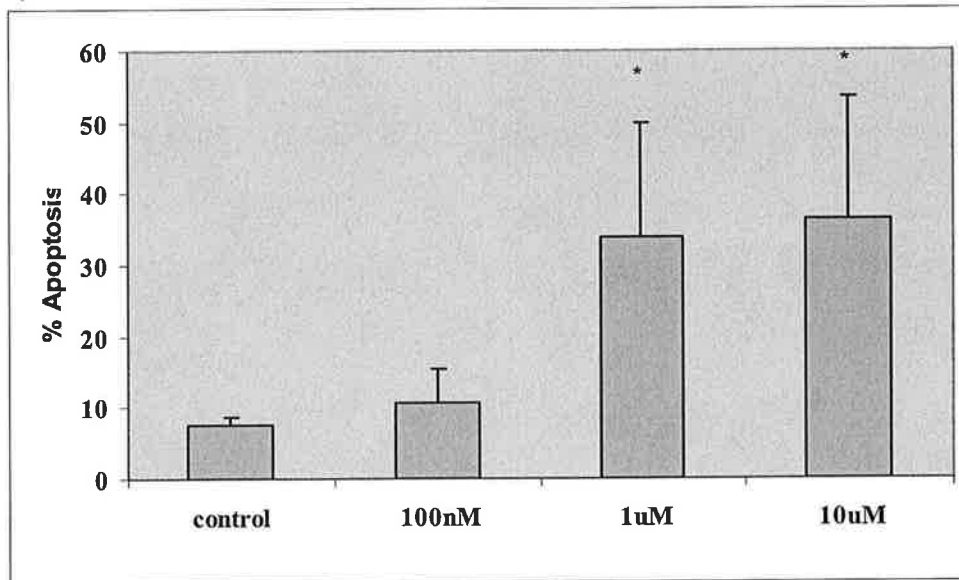


Fig 6.2 Tumour cell proliferation in response to 17AAG

CAL-62 (A) and BHT 101 (B) cells were incubated in 17AAG for 24hr and proliferation measured by spectrophotometer using the MTT assay. Samples were assayed in triplicate, n= 5 individual experiments. Data is recorded as a percentage difference from control and expressed as mean \pm SEM. * denotes a significant difference from control. $p < 0.05$, Anova Scheffe post hoc analysis \$

A)



B)

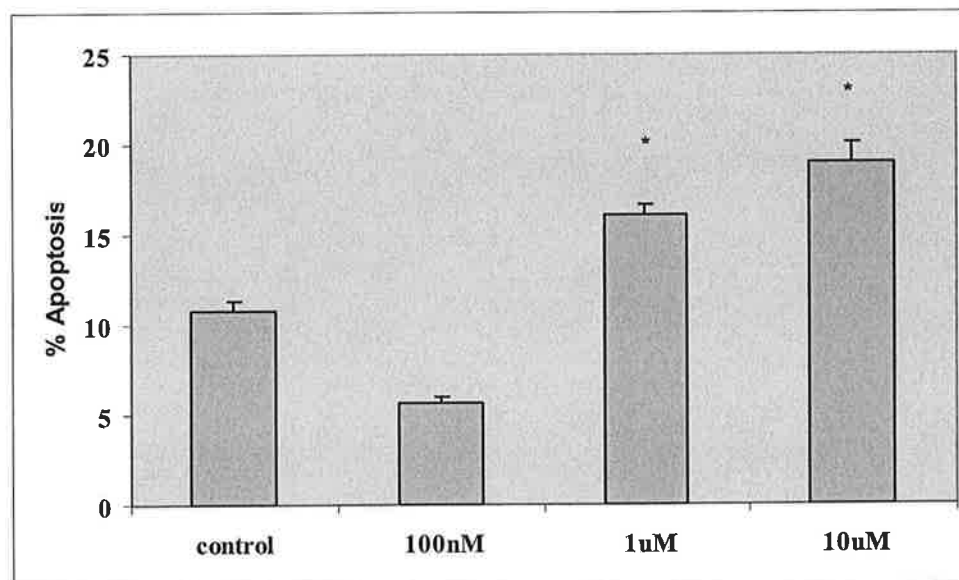


Fig 6.3b 17 AAG induces tumour cell death

CAL-62 (A) and BHT 101 (B) apoptotic cell death was assessed following incubation in 17 AAG for 24hr by measuring Annexin V surface expression. Apoptosis is measured as a percentage of 10,000 cells acquired and expressed as mean + SEM. (n=6 individual experiments. * denotes a significant difference from control. Anova; LSD post hoc analysis.

6.3.2.1 Tumour cell migration.

Metastatic potential was examined following stimulation with VEGF (20ng/ml) for the BHT 101 cells, and EGF (50 ng/ml) for the Cal 62 cells treatment. 17 AAG significantly reduced cell migration at 100 nM and at 1 μ M. VEGF significantly up-regulated tumour cell migration and 17 AAG at 100 nM downregulated this VEGF mediated enhanced tumour cell migration. (Fig 6.4).

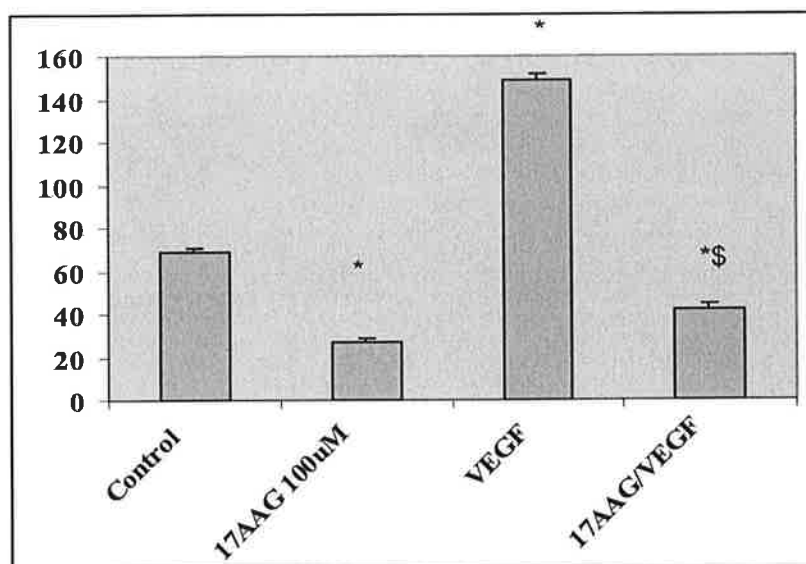


Fig 6.4a Metastatic Potential

Tumour cell cell migration as assessed by transwell assay is graphically demonstrated. 17AAG at 100 nM significantly retard tumour cell migration compared to control in BHT-101 cells. VEGF (20ng/ml) significantly up-regulates tumour cell migration and this stimulation is prevented by 17AAG. Data is recorded as number of cells migrated per high power field (HPF) and expressed as mean \pm SEM. * denotes a significant difference from control. \$ denotes a significant difference from VEGF treated group. $p < 0.05$, LSD post hoc analysis. $n = 3$ independent experiment.

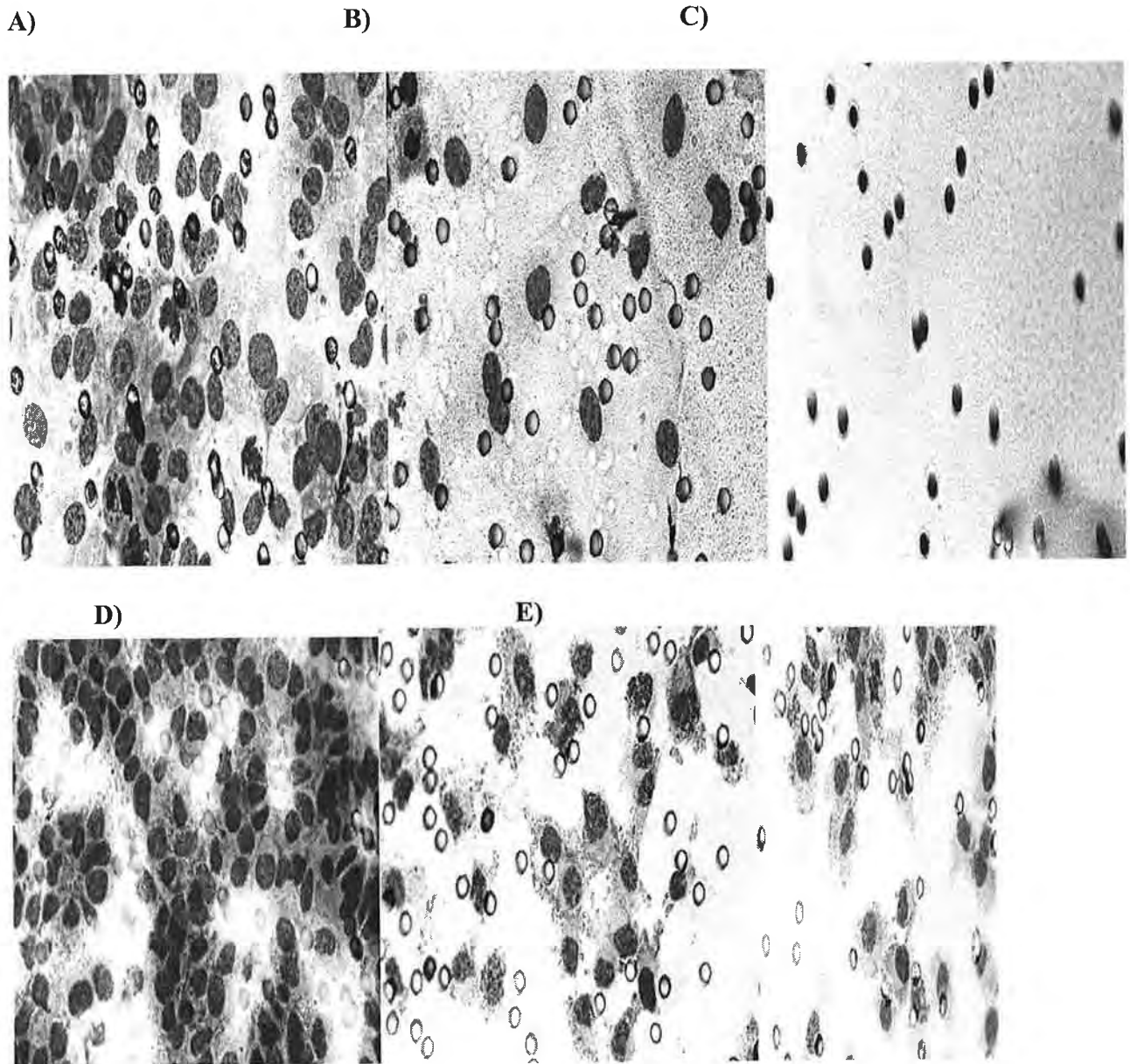
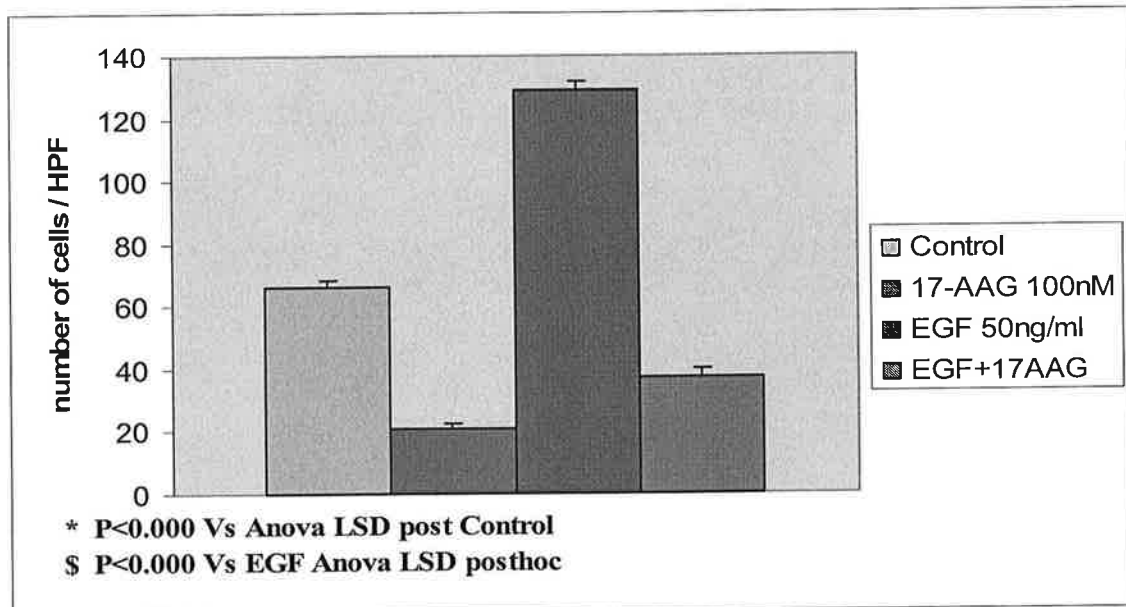


Fig 6.4a **Metastatic Potential**

BHT-101 cells ability to migrate through basement membrane was assessed by transwell assay with 8 μm pores. Migrated cells were stained and counted. The black holes evident on the photographs are the membrane pores. These photomicrographs (x 40) clearly show that 17AAG at 100 nM (B) and 1 μM (C) significantly retard tumour cell migration compared to control (A). EGF (50 ng/ml) significantly up-regulates tumour cell migration (D) and stimulation is prevented by 17AAG (E), (F) ($p < 0.05$, Anova LSD post hoc analysis) $n=3$ independent experiment.

□A)



□B

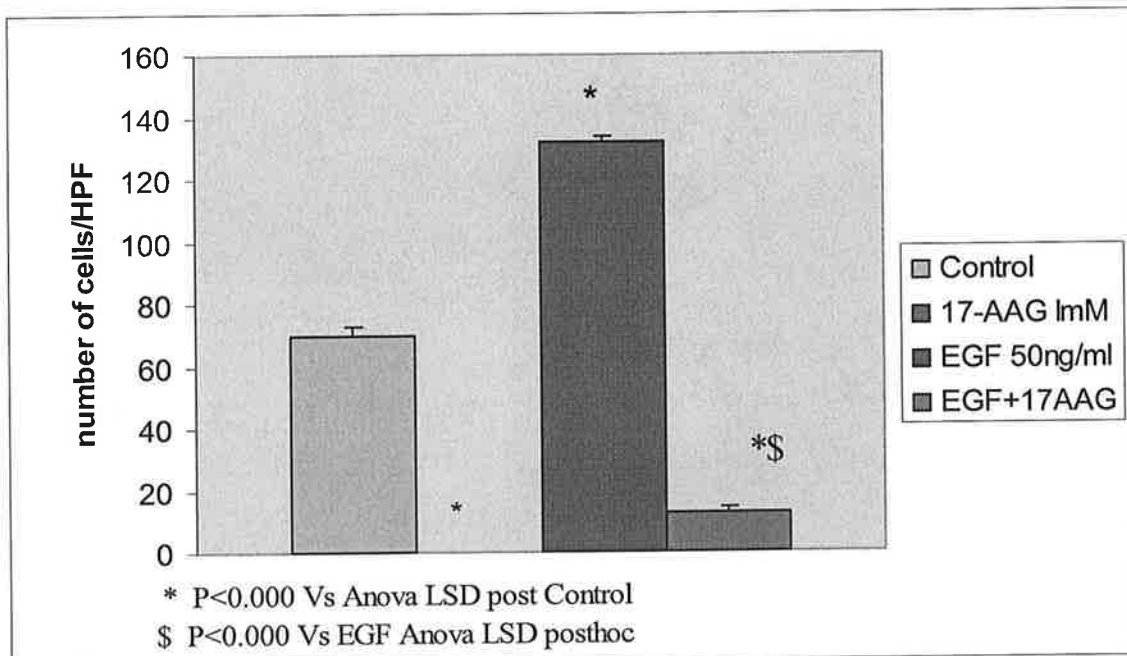


Fig 6.4b Metastatic Potential

Tumour cell cell migration as assessed by transwell assay is graphically demonstrated. 17AAG at 100 nM (A) and 1 μ M (B) significantly retard tumour cell migration compared to control in Cal-62 cells. EGF (50nG/ml) significantly up-regulates tumour cell migration and this stimulation is prevented by 17AAG. Data is recorded as number of cells migrated per high power field (HPF) and expressed as mean \pm SEM. * denotes a significant difference from control. \$ denotes a significant difference from EGF treated group. p<0.05, LSD post hoc analysis. n=3 independent experiment.

6.4 Discussion

We employed the drug 17 AAG. This drug is a less toxic and more stable analog of geldanamycin. Geldanamycin (GA) is a naturally-occurring drug produced by microorganisms to protect themselves from disease-causing substances. Geldanamycin binds to heat shock proteins. All cells produce a common set of heat shock proteins (Hsps) in response to a variety of stresses, including heat, exposure to toxic compounds, or other conditions that cells normally do not experience.

Experiments with bacteria, yeast, fruit flies, and mice have shown that increased production of heat shock proteins can protect an organism against stress-induced damage. There are many known heat shock proteins – each one of them performs a variety of functions that help the cell in both stressful and non-stressful conditions. Most, but not all, heat shock proteins play the role of “molecular chaperones.” Molecular chaperones are substances inside the cell that bind and stabilize proteins at intermediate stages of folding, assembly, movement across membranes, and degradation.

We used this drug at concentrations of 100 nM, 1 uM, 10µM and 100 uM and demonstrated that anaplastic thyroid cells are acutely sensitive to 17 AAG as evidenced by significantly decreased proliferation (chemiluminescence assay) and enhanced cell death (Apoptosis Flow cytometry assay). The metastatic invasive potential of these cells were also observed and their upregulation with EGF stimulation.

In the experiments we have performed we cannot imply our observations are related to an EGFR-17AAG response. Furthermore we did not directly assay for Hsp90 expression in our cells lines. This will require further mechanistic work. In the literature, Wu et al (2009) have investigated 17 AAG in oesophageal cancer. They found HSP-90 to be

expressed by 81 patient oesophageal tumours they examined compared to none or very low levels of HSP-90 expression on normal oesophageal epithelium. Furthermore they demonstrated an interaction between the EGFR and Hsp90 in co-immunoprecipitation experiments. They observed the level of Hsp90 phosphorylation was not sensitive to either 17-AAG or gefitinib treatment. These authors report EGFR expression was down-regulated by 17-AAG treatment in a dose- and time-dependent manner in oesophageal cancer lines and EGF-induced activation of the downstream signaling proteins Erk and Akt was also inhibited by 17-AAG. Furthermore 17-AAG down-regulated the pro-survival IGF-1 receptor and IGF-1-induced Erk and Akt activation. Tyrosine phosphorylation of Hsp90, was detected however, and the functional consequence of Hsp90 tyrosine phosphorylation is not understood to date.

In human thyroid cells Pines et al 2005, demonstrated the direct involvement of APE1/Ref-1 in the regulation of Hsp90 expression, emphasizing the role of this transcriptional coactivator in controlling thyroid cell proliferation.

17AAG as a HSP90 inhibitor displays a 100-fold higher affinity for HSP90 derived from tumor cells compared to HSP90 from normal cells. 17-AAG inhibits Akt activation and expression in tumors and synergizes with a number of antitumor agents such as taxol, cisplatin, and UCN-014. 17-AAG causes the inactivation, destabilization and eventual degradation of HIF-1 α . Banerji et al 2001 recommend 17-AAG exhibits a tolerable toxicity profile with therapeutic plasma concentrations and target inhibition for 24 hours after treatment and some indications of clinical activity at the dose level 450 mg/m²/week and recommend this dose for phase II clinical trials. Ramanathan et al 2005, recommend the dose recommended for future studies at 295 mg /m² weekly x 3,

repeated every 4 weeks. They found that Common drug-related toxicities (grade 1 and 2) were fatigue, anorexia, diarrhea, nausea, and vomiting. Reversible elevations of liver enzymes occurred in 29.5% of patients and hematologic toxicity was minimal.

The epidermal growth factor receptor family is a member of the HER tyrosine kinase growth factor receptor family and is involved in signalling pathways affecting cell growth.

In our immunohistochemical analysis of Anaplastic patient tumour sections two samples were positive, one for Erb-B1 & one for Her-2 positive. Ensinger C et al (2004) assayed twenty-five ATCs, (including 3 ATCs with poorly differentiated thyroid carcinoma parts) were immunohistochemically investigated with a mouse monoclonal antibody directed against EGFR (EGFR pharmDX kit). The anaplastic carcinomas presented with 5 of 25 (20%) without EGFR reaction, 10 of 25 (40%) with reactivity, and 10 of 25 (40%) with overexpression of the receptor. For at least one-third of all anaplastic thyroid carcinomas, EGFR seems to be a promising agent for the targeted molecular therapy of these tumors. In our immunohistochemistry results we expected a higher expression of EGFR to agree with Ensinger et al. Our results may be related to a lack of antigenicity of the tissue.

Nobuhara et al noted in five ATC cell lines; all expressed EGFR and they too suggested EGFR-targeted therapy might be worth further investigation for the treatment of this disease. 17 AAG appears to be directly responsive to concentration of HSP90 within the targeted tissue. Our findings reveal a broad anticancer response and an acute sensitivity of ATC even at low concentration. The apoptotic and anti-metastatic results are of particular relevance. Given the resistant and virulent characteristics this cancer variant

possesses, these results are encouraging and offer potential but further mechanistic work is, of course, required.

The current difficulty from a pharmacological perspective is that 17-AAG has poor water solubility. A hydroquinone derivative with improved water solubility has been developed, 17-AAGH(2). This compound can be oxidised back to 17AAG under aerobic conditions accelerated by Copper which human serum albumin has binding ability. Therefore to prevent this process agents preventing this oxidation will be required as as adjunct to the therapy (Guo et al 2008).

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Chapter Seven

Thesis Conclusion

7.1 Anaplastic Thyroid Cancer (ATC)

This disease, in contrast to differentiated thyroid cancer has a dismal prognosis and remains one of the most aggressive cancers in nature. This body of work has been an exercise in scientific and epidemiology investigation to gain insight into this disease. We questioned the origins and epidemiology profile within Ireland. We revealed in part the immunohistochemical characteristics of human specimens. In laboratory analyses we used cell culture techniques to assess the proliferative and apoptotic influence of two novel chemotherapeutic agents placed upon these cells.

In this thesis we have noted high levels of this disease in comparison to international statistics. Patient numbers may be higher due to the shortfalls in the coding and morphological system. A total of 812 cases of Thyroid Cancer were recorded between 1994-2004 in The Republic of Ireland with 51 cases of Anaplastic Thyroid Cancer. This represents 6.28% of diagnosed thyroid cancers. ATC carries a strong female gender bias. Histopathological patterns of Anaplastic thyroid cancer carry equal prognosis. Given the fact that 20-50% of patients present with metastatic deposits highlights the need for effective systemic chemotherapy. Over 50% of the patients within the Southern Ireland population never smoked however smoking encourages early presentation with smokers presenting a decade earlier.

We analysed the immunohistochemistry on a population of Anaplastic Thyroid Cancer patients examining the expression of the estrogen receptor, EGFR and Her-2.

Takeichi et al 1991 also examined the relationship between the histological grade of dedifferentiation of thyroid cancer and estrogen receptors (ER) immunohistochemically. Six cases of poorly differentiated papillary cancer, 5 (83.3%) were estrogen receptor

negative. We would agree from our results the Estrogen Receptor does not appear to play an important role in this disease with only one of our samples being estrogen receptor positive. Our analysis also found 25% of the tumour specimens were EGFR & Her-2 positive.

We then challenged two cell lines of Anaplastic thyroid cancer, CAL-62, a primary ATC cell line and BHT-101 a metastatic cell line to varying concentrations of Tamoxifen, an estrogen receptor antagonist. We found a response independent to the estrogen receptor. Furthermore Tamoxifen exhibits potent chemotherapeutic potential treating ATC cells in vitro and replication in vivo model. It decreases proliferation, accelerates the induction of apoptosis and downregulates the metastatic, invasive and anti-apoptotic action of Vascular Endothelial Growth Factor. Our results are importantly within acceptable physiological and pharmacological concentrations.

17 Allylamino 17 demethoxygeldanamycin is a tyrosine kinase inhibitor which has potent effects on a variety of cancers. This study demonstrates that anaplastic thyroid cells are acutely sensitive to 17 AAG as evidenced by significantly decreased proliferation and enhanced cell death. The metastatic invasive potential of EGFR expression is also attenuated by 17AAG. Most cancers have multiple genetic aberrations and altered signal transduction pathways. Targetting one of these altered pathways unfortunately has usually failed to achieve successful control of cancer cell growth, because cancer cells can use alternate pathways. Targetting a HSP90 inhibitor with multiple oncoprotein interactions may be a more effective form of chemotherapy. 17AAG is a novel anticancer drug based on differential dependence of HSP90 between cancer cells and normal cells.

Since Kerr first described the process we know apoptosis is a distinct mode of cell death that is responsible for deletion of cells in normal tissues; it also occurs in specific pathologic context of cancer. Morphologically, it involves rapid condensation and budding of the cell, with the formation of membrane-enclosed apoptotic bodies containing well-preserved organelles, which are phagocytosed and digested by nearby resident cells. There is no associated inflammation. A characteristic biochemical feature of the process is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of oligonucleosomal fragments. In many, although not all of the circumstances in which apoptosis occurs, it is suppressed by inhibitors of messenger RNA and protein synthesis. Failure of tumour cell death is a key part of tumour growth. We found the ATC acutely sensitive to both chemotherapeutic agents and recorded using Flow Cytometric Analysis dramatic rates of increased apoptosis.

On diagnosis 90% of ATC patients present with adjacent tissue invasion and 20-50% with distant metastases. Ideally we require the employment of a drug that can target the ATC cells at their most deadly ability, the passage through basement membranes and distant tissue occupation. It would seem likely the penetration of a tumour cell into a basement membrane involves distinct events which include attachment of the tumour cells to the basement membrane via cell surface receptors, secretion of enzymes by the tumour cells which degrades the adjacent membrane and migration of the cells into the target tissue in response to specific chemotactic stimuli.

We performed replication in vivo metastatic studies using fetal calf serum as a chemo-attractant agent and modified our experiments with varying concentrations of chemotherapeutic agent. Blind high power field cell analysis by independent

investigators clearly observed a significant reduction in the metastatic potential of these cells using both chemotherapeutic agents. We reported the significant and potent chemoattractant effect of VEGF on ATC cells and the prometastatic activity the FLT-1 and the FLK -1 receptor expression confers onto these cells. These novel findings merit further investigation, in particular the activity of the less well known FLT-1 receptor, which was thought to be a more benign receptor in its neoplastic ability. The Tamoxifen observations resulted in a dramatic apoptotic and anti metastatic effect independent to estrogen receptor interactions. In conjunction with the high dose cytotoxic regimen described for end stage lung cancer patients this drug has further potential in Thyroid cancer treatment.

In the past 4 years in which time this body of work has been researched and written many advances have been made in Oncology research and in particular the concept of ATC post malignant dedifferentiation. As the first chapter reflected the picture from 2004 we will now examine further insights into this disease up to recent 2009 literature.

Of the 1200 Thyroid Cancer deaths in The United States in 2006, over 50% were due to the anaplastic variant (Are et al, 2006). Global rates of this disease reach 7% of all thyroid cases (Ain et al, 1998). It carries an almost uniformly fatal prognosis.

According to the Surveillance, Epidemiology, and End Results Program of the National Cancer Institute, thyroid carcinomas with follicular phenotype have undergone changing patterns over 30 years (1973-2003). The majority of those affected are within their 6th decade and there is an overall female predominance. Long term survivors are rare.

Thankfully there has been a decline in the incidence of ATC worldwide. The rate of anaplastic carcinoma has decreased by 22%. This may be the result of early identification and successful treatment of papillary and follicular carcinomas (Albores-Saavedra et al, 2007). Improved understanding of the pathogenic and molecular basis for the initiation and progression of this cancer is required. Because of the ineffectiveness of all conventional treatment modalities, novel molecular targeted therapies are required to combat specific molecular targets responsible for the neoplastic phenotype.

Metastatic sites include lungs in 80%, bone 6-15%, and brain 5-13% (Kobayashi et al, 1996). Complete surgical resection is therefore frequently not possible and there is no effective systemic therapy. Prognosis is a dismal 2 to 12 months (Kim et al, 2007, Sugitani et al, 2001). The 10-year relative survival rate for anaplastic carcinoma in patients over 40 years of age was 4.7% (Albores-Saavedra et al 2007). Aggressive multimodal therapy including surgery, radiation, and chemotherapy is recommended for management. The degree of dissemination however highlights the need for effective systemic chemotherapy. There is a lack of prospective treatment protocols, and all evidence in the literature comes from cohort studies for selected patient subgroups or retrospective case series.

Tumorigenesis

ATC tumors are characterized by atypical cells, multinucleated with large bizarre nuclei and multiple atypical mitotic figures. There are no features of differentiated tissue. These are extensively infiltrative and are characterized by the presence of spindle, epithelioid, squamoid giant, or osteoclast like giant cells with frequent admixtures of these cell types in individual tumors (Delellis et al, 2006).

Poorly differentiated carcinoma of the thyroid gland (PDC) are a relatively new classification of thyroid tumors representing a heterogeneous group of epithelial neoplasms with morphologic features and clinical characteristics intermediate between well differentiated and ATC. They are a difficult subset of tumors to confirm from a histopathological perspective and hence this is reflected in the literature. They are however important in the discussion of thyroid tumorigenesis (Saltman et al, 2006).

Thyroid tissue undergoes molecular and genetic alterations, provoking transformation from normal tissue to adenoma and from differentiated to undifferentiated carcinoma. It is now generally accepted that ATC does not arise *de novo* but rather from pre-existing, well differentiated thyroid carcinomas (WDTC) of papillary (PTC) or follicular variant (FTC). The debate at the heart of ATC is this concept of post malignant dedifferentiation (Wiseman et al, 2003).

Thyroid tumorigenesis is complex, involving several cell cycle regulators, oncogenes, growth hormones and cellular differentiation and adhesive compounds. A derangement of the cell cycle and multiple signal transduction pathways results in uncontrolled ATC cellular proliferation and the development of genomic instability (Wiseman et al, 2007, Wiseman et al, 2007).

A wide range in molecular marker expression has been observed by ATC tumors, however, two general groups of specific genetic mutations have been identified. One group of mutations are associated with WDTC and ATC tumors such as BRAF and RAS point mutations (Nikiforova et al 2003, Lemoine et al 1989). Many ATCs with papillary components are derived from BRAF-mutated PTC, because of the addition of p53 mutation (Quiros et al, 2005). Dual association (of WDTC and ATC) would suggest they

are involved with early thyroid tumorigenesis. They are not implicated in the process of post malignant dedifferentiation. Mutations which are believed to be directly involved in the dedifferentiation process are p53 and β -catenin (CTNNB1).

p53

Mutation of p53 are considered to be a late stage of human tumorigenesis (Segev et al, 2003). p53 is mutated or absent in approximately half of all human cancers including lung, colon and breast. 52% of ATC tumors have shown TP53, the gene whose product is p53, or p53 mutation (Lam et al, 2000). p53 plays a key role in its nuclear transcription factor production and regulation of the cell cycle, DNA repair and apoptosis. Either the mutation allows for accelerated genomic instability or loss of wild type p53 results in growth, angiogenesis and the dedifferentiation. To add credence to this theory is the re-differentiation of ATC tissue upon the reintroduction of wild type p53 and the restoration of cellular responsiveness to physiologic stimuli (thyroid stimulating hormone) and re-expression of thyroid peroxidase (Moretti et al, 2000).

p53 and the RET mutation have also been associated. The RET protooncogene located on chromosome 10q11.2 was first identified in 1985. Studies have previously revealed RET rearrangements are associated with papillary carcinomas that lack evidence of progression to poorly differentiated or ATC (Delellis et al, 2006). Transgenic mice, with expression of ret/PTC1, developed PTC that were minimally invasive, and, did not metastasize, however, lack of functional p53 in ret/PTC1 mice has been shown to promote anaplasia and invasiveness of thyroid carcinomas (LaPerle et al, 2000). RET amplification has now however been reported in PTC and ATC. RET amplification was observed in 6 cases of ATC. The frequency of RET amplification-positive cells was

higher in ATC (7.2%-24.1%) than in PTC (1.5%-2.7%). Strong p53 immunoreactivity was related to the highest frequency of RET amplification-positive cells. Amplification of the RET mutation may be induced by a high level of genomic instability, radiation-associated, high-grade malignant potency, and p53 accumulation, in connection with progression of thyroid carcinogenesis (Nakashima et al, 2007).

To counter these findings a study of 62 PDC RET/PTC rearrangements, analyzed by RT-PCR and immunohistochemistry identified in 8/62 or 12.9%. The relatively low prevalence of RET activation in PDC argues against a major role for RET/PTC in the progression from well to poorly differentiated thyroid tumor phenotypes (Santoro et al 2002).

p53 mutation has also been implicated in the over-expression of the serine/threonine kinase Aurora B. This group of cell division regulators (Aurora A, B, C) are responsible for chromosome alignment, segregation, and cytokinesis (Shannon et al, 2002). Normal human thyrocytes express all 3 members of the Aurora kinase family, and their expression is amplified in malignant thyroid cell lines and tissues (Ulissee et al, 2006).

Overexpression of Aurora B results in cell aneuploidy due to disruption of chromosome segregation and chromosome loss. This chromosomal instability is seen in ATC and early reports of blockade induced by RNA interference or by using an inhibitor of Aurora kinase significantly reduced ATC proliferation (Sorrentino et al, 2005).

Cyclins, E-cadherin and β -catenin

Aberrant expression of E-cadherin, β -catenin, and Cyclins may be involved in tumor pathogenesis and metastasis (Lim et al, 2002). Cyclins are proteins that associate with cyclin-dependent protein kinases to regulate their activity and the progression of the cell

cycle through specific checkpoints. Disruption of cyclin action can lead to either cell cycle arrest, or to uncontrolled cell cycle proliferation. Cyclin E is one of the key regulators of the G₁/S transition in the cell cycle.

Overexpression of cyclin E has been observed in several malignancies and is associated with high proliferation, aberrant expression of other cell cycle regulators and chromosomal instability. Overexpression of cyclin E has also been associated with an aggressive tumor phenotype and specific types of *p53* mutations (Barton et al, 2005).

Cyclin D1 is an important regulator of cell cycle progression and can function as a transcriptional co-regulator. The cyclin D1 proto-oncogene is an important regulator of G₁ to S-phase transition in numerous cell types from diverse tissues. Deregulated cyclin D1 degradation appears to be responsible for the increased levels of cyclin D1 in several cancers (Musgrove et al, 2006). Cyclin D1 and Cyclin E have been identified as one of the key therapeutic targets most frequently and most strongly overexpressed in ATC (Wiseman et al, 2007).

E-cadherin and β -catenin are epithelial adhesion molecules in normal epithelium. Loss of E-cadherin - β -catenin adhesion is an important step in the progression of many malignancies. It is mutated or otherwise dysregulated in a variety of human cancers. β -catenin is involved in intracellular signaling and can function as an oncogene, with combination of T-cell factor 4 (Tcf4)-binding site in the promotor region of cyclin D1 and after translocation to the nucleus, transactivates genes. β -catenin mutation are more frequently found in ATC tumors (Fagin et al, 1993, Garcia Rostan et al, 1999).

There is decreased expression of E-cadherin and beta-catenin by the anaplastic tumors when compared with the differentiated thyroid tumors from which they evolved and

derangement of the E-cadherin/catenin complex is associated with the transformation of differentiated into ATC (Wiseman et al, 2006).

Reduced membrane β -catenin has been associated with progressive loss of tumor differentiation. Loss of E-cadherin rather than beta-catenin mutation is implicated for the differentiation 'level' of thyroid carcinomas (Rochas et al, 2003). Low membrane β -catenin expression as well as its nuclear localization or CTNNB1 exon 3 mutation has been associated with poor prognosis (Garcia Rostan et al, 2001).

PI3K/Akt pathway

The phosphatidylinositol 3'-kinase (PI3K) pathway is frequently activated in thyroid carcinomas. Aberrant activation of PI3K/Akt pathway has been shown to play an extensive role in thyroid tumorigenesis, particularly in FTC and ATC, and promotes progression of a thyroid adenoma to FTC and to ATC. Genetic alterations were reported in observed in 25 of 81 (31%) benign thyroid adenoma (BTA), 47 of 86 (55%) follicular thyroid cancer (FTC), 21 of 86 (24%) papillary thyroid cancer (PTC), and 29 of 50 (58%) anaplastic thyroid cancer (ATC), with FTC and ATC (Hou et al, 2007).

Mutant PIK3CA is likely to function as an oncogene among ATC. In 18 of the 20 ATC cases showing coexisting differentiated carcinoma, mutations, when present, were restricted to the ATC component and located primarily within the kinase domain. Activation of Akt has been observed in most of the ATC with PIK3CA mutation and is therefore a therapeutic target (Garcia- Rostan et al, 2005).

EGFR, VEGF

The Epidermal Growth factor receptor (EGFR) is a cell membrane receptor that plays a key role in cancer development and progression. Increasing evidence has shown that the

over-expression of EGFR closely correlates with advanced tumor stage and metastasis, and poor clinical outcome in many human cancers including breast, cervix, lung, bladder and head and neck. EGFR overexpression is a common finding in ATC in at least one-third of all anaplastic thyroid carcinomas (Ensinger et al, 2004). EGFR targeted therapies have now attracted increasing attention in the literature. There are many kinds including antireceptor monoclonal antibodies, antiligand monoclonal antibodies, ligand-toxin conjugates, scFv-toxin conjugates, ligand-genistein conjugates and tyrosine kinase inhibitors.

VEGF plays an important role in thyroid carcinogenesis and cancer progression. The potential implications of VEGF in the progression of thyroid neoplasms appear to be diverse for each histologic subtype. Viglietto et al first demonstrated that expression of VEGF is positively associated with tumorigenic potential of thyroid cancer cell lines (Viglietto et al, 1995). VEGF protein expression was higher in cancer cell lines and a few primary tumours than in primary cultures of normal thyroid cancer cell lines. Those tumours with high VEGF content had a high index of cell proliferation. VEGF is implicated in angiogenesis and metastases as the initiation of a vascular phase marks a period of accelerated growth, local invasion and ultimately metastases of thyroid neoplasms. Dual inhibition of EGFR and VEGFR signaling pathways in tumor cells and tumor-associated endothelial cells in combination with chemotherapy can provide a new approach to treatment.

Cetuximab and bevacizumab (monoclonal antibodies against EGFR and Vascular Endothelial Growth Factor (VEGF)) alone and in combination inhibit tumor growth and angiogenesis in an in vivo model of ATC (Prichard et al, 2007). This therapy was also

compared and found to be superior to doxorubicin. Cetuximab with Irinotecan, a topoisomerase inhibitor in combination inhibit the growth and progression of orthotopic ATC xenografts in nude mice (Kim et al, 2006).

AEE788 is a dual kinase inhibitor of EGFR and VEGF. It has shown promising anti-proliferative and pro-apoptotic potential (6-8 fold increase) on ATC cell lines when administered alone or in combination with paclitaxel. The microvessel density within the ATC xenografts was decreased by >80% compared with the control group. Immunofluorescence microscopy revealed EGFR autophosphorylation inhibition on ATC cells as well as inhibition of VEGFR-2 autophosphorylation on tumor endothelium (Kim et al, 2005)

Evaluation of the role of Cetuximab and AEE 788 on growth, apoptosis, and autocrine VEGF-secretion revealed Cetuximab consistently decreased VEGF secretion but did not affect tumor cell proliferation in vitro. In contrast AEE 788 not only reduced VEGF secretion but also exhibited a dose-dependent inhibition of tumor cell proliferation and was a potent inducer of apoptosis. These effects were accompanied by decreased levels of pEGF-R, pVEGF-R, pAkt, and pMAPK (Hoffman et al, 2007).

AEE788 is an exciting therapeutic option and impressive animal study results for colon cancer warranted further investigation, alone and in combination for ATC (Yohoi et al, 2005).

Sorafenib is a multikinase inhibitor of the BRAf, CRAf, C-KIT, flt3, VEGFR-2, and PDGFR-beta kinase and has shown a survival advantage in renal cell carcinoma and hepatocellular carcinoma. In ATC sorafenib has shown activity which translated into a survival benefit in nude mice models (Kim et al, 2007).

Sodium-Iodide symporter and I^{131} therapy

The sodium iodide symporter (NIS) is an intrinsic plasma membrane protein that mediates thyroid follicular cells to actively transport iodide into the thyroid gland and a number of extra thyroidal tissues.

NIS-mediated iodide accumulation allows diagnostic thyroid scintigraphy as well as effective therapeutic application of radioiodine in benign and malignant thyroid disease. These functions are lost in ATC due to decreased expression of NIS and hence a loss of iodide accumulation. Targeting NIS expression in ATC cancer cells, enables these neoplastic cells to concentrate iodide from plasma and in so doing offer the benefits of radioiodine therapy.

Transfection of human sodium-iodide symporter, (hNIS) into hNIS-deficient follicular thyroid carcinoma cell line restored the in vivo iodide accumulation in xenografted tumors and their susceptibility to radioiodide therapy. Conventional conditioning with thyroid ablation and low iodide diet can improve iodide kinetic (short half life) characteristics resulting in a therapeutic response. However, to achieve sufficient radioiodide tumor doses for therapy, further strategies are necessary, aiming at the mechanisms of iodide efflux in particular (Smit et al, 2007).

ATC cells (wild type), have been transfected with hNIS. Stable transfected cells express hNIS on the cell membrane and accumulated 87-fold and 4.4-fold radioiodide in vitro and in vivo compared to wild-type cells. Administration of a therapeutic dose of I^{131} into these mice bearing tumors, effectively inhibited tumor growth as compared to control mice (Hsieh et al, 2007). Further studies have identified the possibility of imaging and therapy using NIS gene transfection in ATC, although the short retention time is

considered the major impediment to be resolved for the successful implementation (Lee et al, 2004). One method of improving this function has been the use of 1-microM, all-trans retinoic acid (tRA). Treatment of ATC cells with tRA expressing the NIS gene significantly elevated their NIS-mediated radioiodine uptake ability (Jeong et al, 2006).

The thyroid transcription factors TTF-1 and Pax8 cooperate in the transcriptional activation of thyroid-specific genes such as thyroglobulin (Tg), thyroperoxidase (TPO), and sodium/iodide symporter (NIS). ATC cells transfected with a Pax8 gene expression vector showed re-activation of several thyroid specific genes including NIS, Pendrin, Thyroglobulin, TPO and TTF1. In ARO-Pax8 clones NIS protein was also localized both in cell cytoplasm and membrane. Thus, a re differentiation ability to uptake the radioiodine was partially restored. Expression of Pax8 also conferred a reduced rate of cell growth (Presta et al, 2005). Whilst experimental, these therapies potentially offer the greatest therapeutic opportunity for this disease.

Multidrug resistance

A major form of resistance exhibited by ATC cells against a wide variety of antineoplastic agents currently used involves the extrusion of cytotoxic molecules by a group of membrane proteins (Sugawara et al, 1995). This prevents intracellular drug concentrations reaching a threshold of cell destruction. The ABC family of ATP binding cassette proteins mediate the translocation of various molecules across membrane barriers. ABC protein consists of membrane-embedded transmembrane domains (TMD) and ATP binding domains (ABC). The transmembrane regions anchor the protein to the membrane and form a pore through which the transport of a large variety of substrates occurs. Drug extrusion mediated by these primary active transporters is driven by the

energy of ATP hydrolysis. The P-glycoprotein (MDR-1) and multi-drug resistance protein 1 (MRP1) recognize hydrophobic substrates within the membrane bilayer or in its vicinity, and this type of recognition makes these proteins highly effective pumps, preventing the cellular entry of toxic compounds (Yasuhisa et al, 2007). Analysis of four ATC tumors revealed intrinsic drug resistance to 9 out of 11 antineoplastic agents. MRP mRNA was detected by reverse transcriptase-polymerase chain reaction, but not MDR-1 and MDR-3 mRNAs. This indicates that the multidrug resistance is mediated by a P-glycoprotein-unrelated mechanism. MRP-1 inhibition has previously been performed using indomethacin analogues potentiating the toxicity of a number of chemotherapeutic agents (Touhey et al, 2002).

Conventional therapy

Surgery remains an important component of the multimodal therapy and is commonly adopted as primary treatment. In a recent retrospective report on the clinical outcome of 30 ATC patients, treatment arms were surgery followed by adjuvant-combined chemoradiotherapy, neo-adjuvant chemoradiotherapy followed by surgery and adjuvant chemotherapy or, chemotherapy alone. Surgical intervention was broken into 'maximal debulking' or 'palliative resection'. Maximal debulking entailed total or near-total thyroidectomy and complete resection of all gross tumor or minimal residual disease adherent to vital structures. This was performed independently of the presence or absence of distant metastases. Palliative resections left macroscopic residual disease in the neck. Using the treatment arm of maximal debulking followed by adjuvant chemoradiotherapy modified survival. This was the only treatment arm that improved survival (Brignardello et al, 2007).

Age < 60 years, female gender, intrathyroidal tumor, external beam radiotherapy, surgical resection, and combined surgical resection of tumor and radiotherapy are associated with a lower cause-specific mortality (Kebebew et al, 2005). There are differing prognostic factors quoted in the literature such as age < or = 70 years, absence of dyspnoea or dysphagia at presentation, a tumor size < or = 5 cm, and any surgical resection improved survival (Pierie et al, 2002). Tracheostomy is now performed for impending airway obstruction but is not a prophylactic strategy.

A regime of preoperative hyperfractionated radiotherapy and doxorubicin followed by surgery when feasible has been documented to achieve local control and avoid tracheostomy for ATC patients. Radiotherapy has evolved to a combination of preoperative and postoperative treatment and using higher doses, along with hyperfractionating and accelerating dose schedules. Chemotherapy has changed from monotherapy to combination therapy, and newer drugs combinations of vinorelbine/gemcitabine and paclitaxel/gemcitabine have exerted a trend of synergy (Are et al, 2006, Lang et al, 2007, Voight et al, 2005).

Other targets under investigation include proteasome inhibitors, e.g. bortezomib, matrix metalloproteinase inhibitors, e.g. minocycline, farnesyltransferase inhibitors, e.g. manumycin, estrogen metabolites, peroxisome proliferator activated receptor (PPAR γ) agonists, histone deacetylase inhibitors, e.g. valproic acid, TRAIL receptor (TNF-related apoptosis-induced ligand), and others (She et al, 2006, Roswall et al 2006, Miccoli et al 2006, Mitsiades et al, 2005).

7.2 Conclusion

The origins and treatment options of this disease are yet to be fully elucidated. Etiologic studies addressing temporal changes in reproductive factors, more intensive diagnostic activities, and changes in histological criteria are warranted to include those cancers under the title of 'undifferentiated thyroid' but not statistically included within the anaplastic histopathological category within this country. This will allow a more accurate representation of this disease as currently the estimated figures are conservative.

The results of further research into this disease we believe will have major knock-on implications for oncology as a broad subject and those unfortunate patients who currently have little option apart from a palliative course. The only true successful clinical reports to date come from patients with differentiated tumours with *a focus* of ATC found within these tumours rather than a fully established ATC tumour.

We are moving into a new era of Cancer treatment where chemotherapy will not be given to a patient independent of the characteristics of the tumour but rather selected therapies will be administered to react with specific molecular targets. In time the greater understanding of the factors responsible for the biological translation of cancer aggression and metastatic activity will be understood. Targeted molecular therapies are proving to be the recent advances in Thyroid cancer management. (Stenner F et al, 2008 Rovere et al, 2008)

ATC remains a devastating disease for these unfortunate patients and their families. The National Institutes of Health lists seven treatment trials currently recruiting. Several of the agents outlined above are under investigation. Clinical trials are paramount in this rare devastating disease and success will have significant implications for the treatment of

many other cancers. Multidisciplinary care and combination therapeutic strategies are required. Progress has been made understanding the cellular processes governing the mechanism of post malignant de-differentiation and the metastatic process. This has brought a variety of novel therapies of sufficient merit to conduct further phase trials. We believe this thesis offers fresh insight into all aspects of this disease which we hope, in part, may offer new clinical investigators a variety of options for oncology study. We also believe a very positive development has been the formation of The IHNOD database. This represents a significant commitment to Head and Neck Oncology Epidemiology and Patient Management in Ireland.

Anaplastic thyroid cancer remains a worthy disease for continued focused research in the Otolaryngology, General Surgery and wider Oncology Scientific communities.

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Domestic Presentations related to this Thesis

The Royal College of Surgeons Research Day

Dublin, March 30th 2005, Oral Prize Session

'Tamoxifen as a novel chemotherapeutic agent in anaplastic thyroid cancer'

Sir Peter Freyer Surgical Symposium

Galway, September 2005, Poster Presentation

'A changing role for Tamoxifen'

Irish Otolaryngology Society

Westport, Co Mayo, October 2005

'Tamoxifen induces tumour cell apoptosis and blocks metastatic invasion in Anaplastic Thyroid Cancer'

Royal Academy of Medicine

April 2006, Mount Juliet, Co Kilkenny

'Pro-metastatic Protein Expression and their functional inhibition using 17-Allylamino-17-demethoxy-Geldanamycin (17AAG) in Anaplastic Thyroid Cancer'

The Irish Otolaryngology Society

Slieve Russell Hotel, Co Cavan, October 2006

'Anaplastic Thyroid Cancer, The Irish Experience'

The Sylvester O'Halloran Surgical Symposium 2007

University of Limerick, March 07

'The role of novel chemotherapeutic drugs in the treatment of Anaplastic Thyroid Cancer'

The Sylvester O'Halloran Surgical Symposium 2007

University of Limerick, March 08

'Immunohistochemical insights in Anaplastic Thyroid Cancer'

International Presentations

Otolaryngology Research Society (ORS) 2005

Grays Hall, London, England Sept 30th 2005

'Tamoxifen induces tumour cell apoptosis and blocks migration in Anaplastic thyroid cancer (ATC)'

SARS Society of Academic & Research Surgery

Annual Meeting at The Royal College of Surgeons of Edinburgh, Scotland

13 January 2006

'Tamoxifen induces tumour cell apoptosis and blocks migration in Anaplastic thyroid cancer (ATC)'

The American College of Surgeons Meeting 2006

'Tamoxifen induces tumour cell apoptosis decreases proliferation and blocks metastatic invasive potential in Anaplastic Thyroid Cancer (ATC)'

October 2006, Chicago, USA

The American College of Surgeons Surgical Research Meeting

Massachusetts Chapter, Boston. December 2007

'The Role of Tamoxifen in Anaplastic Thyroid Cancer'

Winner of Poster of Distinction

Publications (to date)

'Anaplastic (undifferentiated) thyroid carcinoma, improved insight and therapeutic strategy into a highly aggressive disease'

JP O'Neill, B'ONeill, M Walsh, D Bouchier Hayes.

The Journal of Laryngology & Otolology 2005;119:585-591

'Tamoxifen as a novel chemotherapeutic agent treating anaplastic thyroid cancer'

JP O'Neill, C Condron, AM Byrne, M Walsh, D Bouchier-Hayes

***European Journal of Cancer* ECCO Supplement 1.096 Abstract publication**

'Tamoxifen induces tumour cell apoptosis and blocks migration in Anaplastic Thyroid Cancer

JP O'Neill, C Condrón, AM Byrne, M Walsh, D Bouchier-Hayes
Irish Journal of Medical Science 2005;174(3):27 Abstract publication

'Tamoxifen induces tumour cell apoptosis and blocks migration in Anaplastic thyroid cancer (ATC)'

JP O'Neill, C Condrón, AM Byrne, M Walsh, D Bouchier-Hayes
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Appendix 2

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Re: Use of NCRI data for PhD thesis, Paul O'Neill

Dear Paul:

The National Cancer Registry acknowledges that you have reviewed its data on anaplastic thyroid cancer cases in Ireland and will use the results of analysis carried out on this data in your PhD thesis.

We wish you every success with your studies.

Kind regards,

Sandra Deady, PhD
Data Analyst, NCRI

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