Effect of Estrogen on the Bcl-xL Expression and the Proliferation of Thyroid Papillary Carcinoma Cells

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Abstract

There are a number of predisposing factors in the development of human thyroid carcinoma, but the underlying pathogenesis of thyroid carcinoma remains unknown. One of the specific features of thyroid cancer is its predilection for women of reproductive age relative to men. The incidence of thyroid tumors and carcinomas is three times more frequent in females than males. An elevated risk was also documented in women who used estrogens for gynecological problems, but not for low dose estrogen replacement therapy in postmenopausal women. The presence of estrogen receptors in thyroid tissues was demonstrated by immunohistochemical method. In some studies, higher levels of ERs were found in neoplastic thyroid tissues but not in normal thyroid tissues. These epideminological and experimental studies indicate that estrogen may play a pathological role in thyroid carcinoma.

In this study, we investigated the effect of estrogen (estradiol, E2) on apoptosis in the well-differentiated human thyroid carcinoma (KAT5) cell. The KAT5 cells were treated with 10^{-11}, 10^{-9}, 10^{-7} M/L E2 for 24 and 48 hours. We examined the cell proliferation of the E2 treated KAT5 cells and compared it with that of the testosterone treated KAT5 cells by MTT assay. The growth effect of E2 was also attenuated by tamoxifen. We have studied the expression of the estrogen receptor (ER), anti-apoptotic Bcl-xL and pro-apoptotic Bax protein and mRNA levels in KAT5 cells by western blot and RT-PCR analysis. The protein expression of the anti-apoptotic potential (Bcl-xL/Bax ratio)

increased in the dose-dependent manner. The results indicate that the mechanism of action of estrogen on KAT5 cell growth may be associated with up-regulation of Bcl-xL gene at both mRNA and protein levels.

中文摘要

促使人類甲狀腺癌生長的因素有很多，但其主要致病原理仍然不明。甲狀腺癌的發病率在婦女明顯高於男性，而尤其是在生殖年齡期的婦女。有文獻指出，有婦科問題的婦女使用雌激素可增加發病的危險,但此並不發生於少量雌激素代替治療的使用者。在一些研究中，免疫組織化學技術證明甲狀腺組織擁有雌激素受體，甲狀腺癌瘤組織的雌激素受體表達量更比正常組織的多，綜合這些流行病理及實驗室研究指出雌激素可能是引致甲狀腺癌的原因之一。

在本項研究中，我們檢測雌激素（雌二醇）對人類甲狀腺癌細胞(KAT5) 的細胞生長及細胞凋亡的影響。KAT5 細胞經 10^{-11}, 10^{-9}, 和 10^{-7} M/L 雌二醇治療 24 和 48 小時後，我們利用 MTT 分析測試雌二醇對細胞生長的影響，同時也比較了其與雄激素對細胞生長的影響的不同。雌二醇對細胞生長的影響可被 tamoxifen 所減輕。另外，我們用 Western blot 和 RT-PCR 分析測試雌激素受體，抗凋亡 Bcl-xL 和促凋亡 Bax 蛋白及 mRNA 在.細胞的表達狀況。雌激素雖對 Bax 的表達無顯著的影響,但可明顯增加 Bcl-xL 的水平，因而，雌激素可以劑量依靠方式增加抗凋亡潛勢 (Bcl-xL/Bax 比率)。總括結果，雌激素對甲狀腺癌 KAT5 細胞成長具有抑制作用,此抑制作用可能與其提升 Bcl-xL 的表達有聯。

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Lee ML, Chen GG, Vlantis AC, van Hasselt CA

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Abbreviations

DNA	deoxyribonucleic acid
DTT	dithiothreitol
E2	estradiol
ECL	enhanced chemiluminescene
ER	estrogen receptor
FBS	fetal bovine serum
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PMSF	phenylmethylsulfonyl fluoride
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TBST	tris-buffered saline with tween 20
Tam	tamoxifen

CONTENTS

CHAPTER THREE: RESULTS

CHAPTER ONE: INTRODUCTION AND LITERATURE

1.1 THYROID CANCER AND ITS EPIDEMIOLOGY

Thyroid cancer is a relatively rare cancer accounting for approximately 1 % of all cancers in worldwide (Correa *et al.,* 1995). However, it is the highest incidence of cancer of endocrine gland. The incidence rate shows the striking worldwide similarities in the female excess of thyroid cancer in a specific age group.

1.1.1 Histology

Cancer of the thyroid is comprised of four primary histological types. Anaplastic cancers, which also arise from follicular cell, are undifferentiated. They occur primarily in the elderly, and are associated with a much poorer prognosis. Medullary cancers arise from the calcitonin-secreting parafollicular cells. Papillary and follicular cancers are the well-differentiated thyroid cancers arise from follicular cells, and they occur primarily in young to middle-aged women (Correa *et al.*, 1995). They are the most common histological types of thyroid cancer. For instance, among all thyroid cancer cases diagnosed between 1998 to 1999 and identified in the Hong Kong cancer registry (Table 1), the distribution by histology was 77.4 % papillary, 12.6 % follicular, 1.8 % medullary, and 2.4% anaplastic, with the remainder

categorized as "others". The histological distribution is similar in Japan and United States (Koike *et al.*, 1991 and Gilliland *et al.*, 1997).

Histology	Male	%	Female	%	Total	%
Papillary carcinoma	140	74.9	467	78.2	607	77.4
Follicular carcinoma	19	10.2	80	13.4	99	12.6
Medullary Carcinoma	5	2.7	9	1.5	14	1.8
Anaplastic Carcinoma	12	6.4	7	1.2	19	2.4
Other specified carcinoma	4	2.1	4	0.7	8	1.0
Unspecified carcinoma	1	0.5	7	1.2	8	1.0
Other specified morphology	1	0.5	-	0.0	1	0.1
*Unspecified morphology	5	2.7	23	3.9	28	3.6
Total	187	100	597	100	784	100.0

* "Unspecified morphology" includes all cases that have no microscopic verification of diagnosis or the results are not interpretable.

Table 1. Thyroid Cancer Histological subtype in Hong Kong (1998-1999). (Hong Kong Cancer Registry).

1.1.2 Gender comparison

Throughout the world, age-adjusted incidence rates for thyroid cancer show 2-to 3-fold excess among females relative to males (Figure 1). The female: male ratio varies by age and histology. Age-specific incidence data from Los Angeles County (Figure 2) show that the female: male rate ratio is highest from ages 15 to 40, ranging from 3.1 to 6.0. The female excess remains approximately double from age 40-55 and declines thereafter. Among almost 12,000 cases of thyroid cancer diagnosed among whites in 1973-1987 and identified in the United States Surveillance, Epidemiology, and End Results cancer registry (SEER), the female: male ratio was 2.6 for papillary cancer, 2.2 for follicular cancer, 2.0 for medullary cancer, and 1.0 for anaplastic cancer (Correa *et al.,* 1995). This predilection for women of reproductive age phenomenon makes a hint on the pathogenesis of thyroid cancer. In their reproductive age, normal females have a range of 180-1500 pmol/l endogenous estrogen in the blood circulation. After the reproductive age, the endogenous estrogen level is lowered to less than 200 pmol/l in the circulation system of the female. In contrast, the endogenous estrogen level is less than 300 pmol/l in men. Therefore, estrogen has been suspected as one of the factors that contribute to the development of this female dominant disease, thyroid cancer.

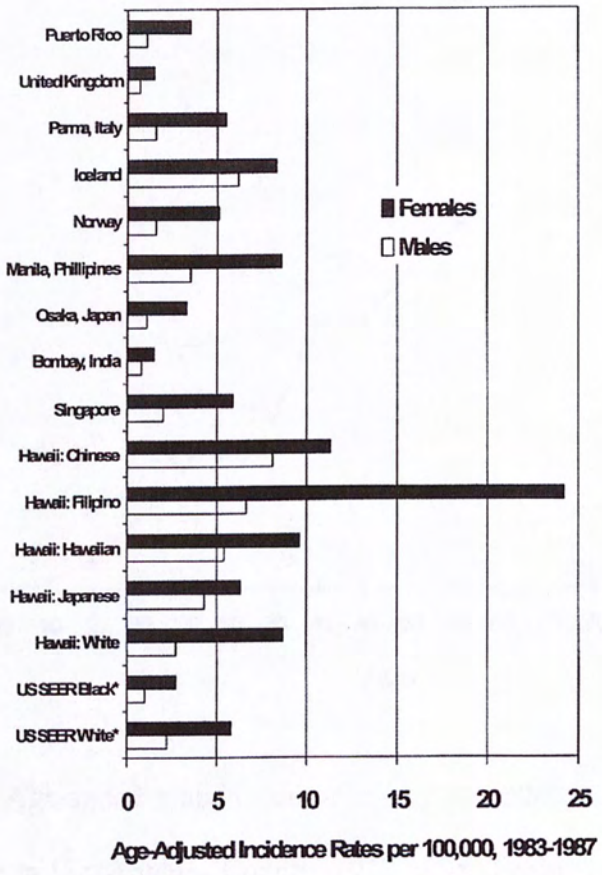


Figure 1. Age-adjusted incidence rates (per 100,000) of thyroid cancer, 1983-1987 (Parkin *et al.*, 1992).

*SEER=Surveillance, Epidemiology, and End Results cancer registry

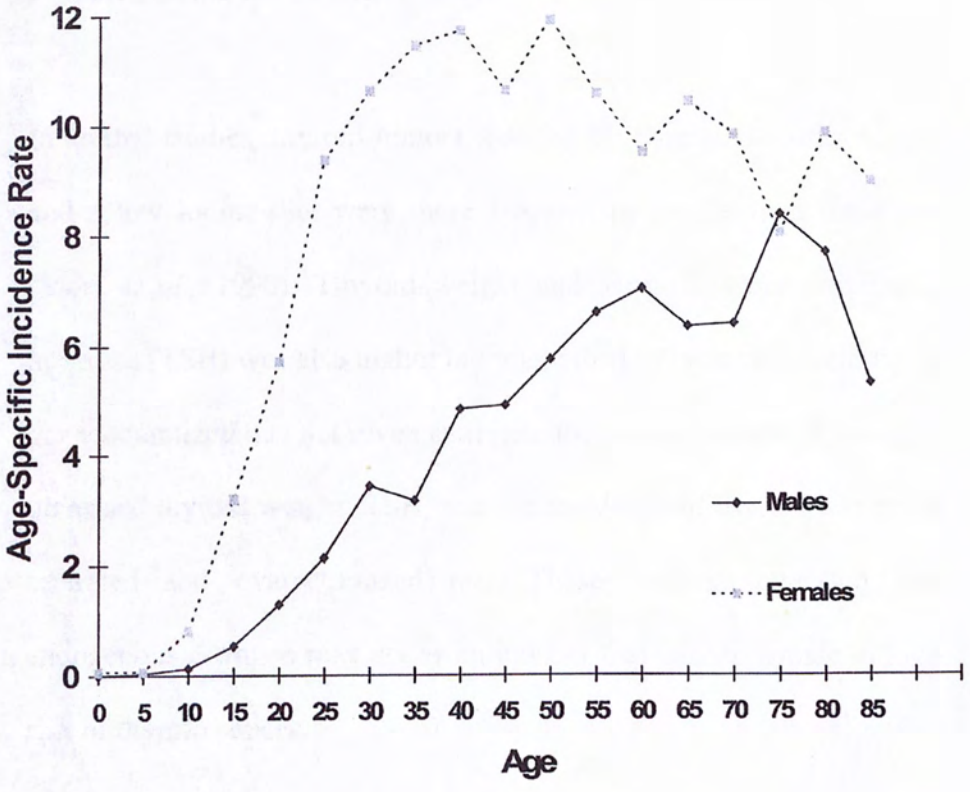


Figure 2. Age-specific incidence rates (per 100,000) of thyroid cancer by gender in Los Angeles County, 1972-1995 (Fagin, Thyroid Cancer. 1998).

1.1.3 Female Hormone – A Risk Factor

In animal studies, thyroid tumors induced by N-methyl-N-nitrosourea and a low iodine diet were more frequent in female than male rats (Mori *et al.,* 1990). Thyroid weight and serum thyroid-stimulating hormone (TSH) was also higher in female than in male rats. Relative to gonadectomized rats not given estrogen, the administration of estrogen increased thyroid weight, TSH, and the incidence of thyroid tumors in castrated and ovarectomized rats. These studies suggested that endogenous estrogen may act as an inducer that causes female in high risk of thyroid cancer.

Furthermore, the incidence of thyroid carcinoma in women who take oral contraceptives has moderately elevated risk than in those who do not (McTiernan *et al.,* 1984). An elevated risk was also documented in women who used estrogens for gynecological problems, but not for low dose estrogen replacement therapy in postmenopausal women (Ron *et al.* 1987 and Persson *et al.* 1996). These epidemiological studies indicate that exogenous estrogen may also promote the development of thyroid tumors.

1.2 BIOLOGICAL BACKGROUND OF SEX HORMONES

The process of growth regulation in multicellular organisms is under the control of various signals. Among those signals, hormone is one of the known factors to regulate cell proliferation. Hormone is a chemical substance that is released locally via a paracrine pathway or systematically via the circulation system. Hormone is usually produced in small amounts but it elicits a typical physiology response in its target cells (Figure 3). Hormone usually binds its receptor with selectivity to initiate the specific response in the target cell.

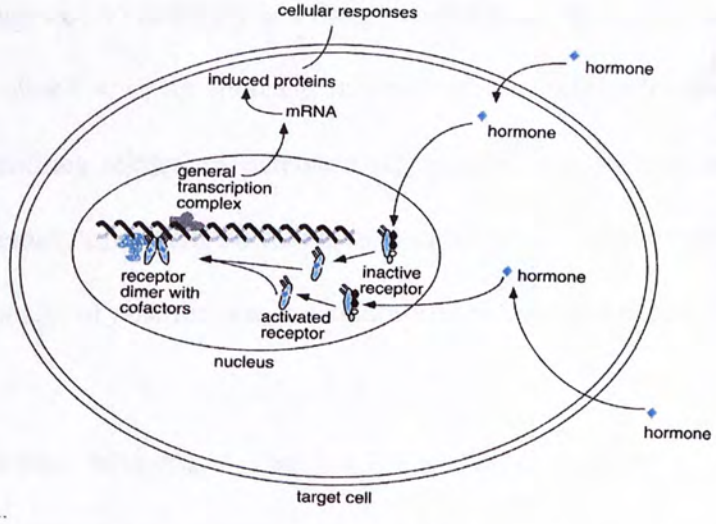


Figure 3. General action of steroid hormone. Steroid hormone passes through the plasma membrane and binds to its intracellular receptors. Hormone binding activates the receptor, which forms complexes with other proteins and binds to specific sites on DNA to initiate transcription and subsequently to induce the protein production. (Goodman 2003)

1.2.1 Estrogen

Estrogen (E2) is a steroid hormone that is traditionally connected with female reproduction system. It is mainly synthesized in the ovary, but also in peripheral tissues through aromatization of androgen (Cooke *et al.* 1998). It directs the development of the female phenotype in embryogenesis and during puberty by regulation of gene transcription and, thus, protein synthesis (Beato 1989). It also induces the production of gonadotropins, which, in turn, induce ovulation. The level of estrogen production is started to decrease in women between the ages of 45 and 55 years during menopause. Estrogen deprivation is associated with an increase in level of total cholesterol, the risk of disabilities related to osteoporosis, genital atrophy and etc. It is an extremely important hormone used in the clinic for the prevention and treatment of postmenopausal symptoms and as contraceptives.

However, estrogen is also known as a mitogen that stimulates cell proliferation and prevents cell death in many cell types. In patients, estrogen is known to stimulate breast and uterus cancer development. It is also been shown to prevent apoptosis through an estrogen receptor (ER)-mediated mechanism (Thompson 1994). The mitogen-activated protein (MAP) kinase (Song *et al.* 2002), phosphoinositide 3-kinase (PI3-kinase) (Sun *et al.* 2001) and many cell cycle-regulating genes

(Lodenhofer *et al.* 2000) are known to be involved in the action of estrogen.

1.2.2. Estrogen antagonist

Tamoxifen (Tam) is a non-steroidal selective estrogen receptor modulator that inhibits the action of estrogens. It is a mixture of estrogen agonist and antagonist, which suppresses tumor growth. It has been used in endocrine therapy for many years to treat breast cancer patients. The effect of estrogen and tamoxifen on the growth of estrogen-dependent breast cancers has been intensively studied. Study indicates that the mechanism responsible for the pharmacological effect of Tam is related to its ability to bind to estrogen receptors and to form a Tam-estradiol receptor complex, which bind to the nuclear binding sites on the genome (Pienta *et al.* 1995). The binding prevents the receptors from recycling thereby reducing the number of receptor molecules available for subsequent estradiol activity (Goodman and Gilman 1985 and Jordan *et al.* 1977). Therefore, the mitogenic and survival effects of estrogen have been further confirmed by observations that the anti-estrogen tamoxifen block breast cell proliferation (Budtz *et al.* 1999 and Spyridopoulos *et al.* 1997).

1.2.3. Estrogen receptors

Estrogen activity is mediated by its cellular receptors (ER). Binding of E2 to the ER induces a conformational change that enables the ER to recruit transcriptional coactivitors and to induce the expression of estrogen-regulated genes (Beato, 1989). To date, two isoforms of the ER (α and β) (Figure 4) have been identified and both subtypes are able to bind to DNA as homo- or heterodimers (Pace *et al.*, 1997). ERs are found in reproductive tissues and higher centers in the central nervous system that are involved in reproduction. However, they are also expressed in non-reproductive tissues, like thyroid and endothelium (Kawabata *et al.* 2003, Macpherson *et al.* 1999). Changes in the level of sex steroids are associated with the expression of their respective receptors. In endothelial and breast tissues, the expression of ER isoforms appears to be up regulated by 17beta-estradiol during the menstrual cycle. But this effect appears to be tissue- or cell- specific (Zou *et al.* 1998, Santagati *et al.* 1997).

The expression of ERα is not detected in the normal thyroid gland, but the expression of ERβ is detected throughout the development of the normal thyroid gland in a study by Kawabata *et al.* ERβ immunoreactivity was detected throughout the development of human thyroid gland from 11 weeks pregnancy when the large quantity of estrogen was produced in placental. The result suggested that estrogen may be involved in the

development of fetal thyroid gland and these effects may be mediated via ERβ.

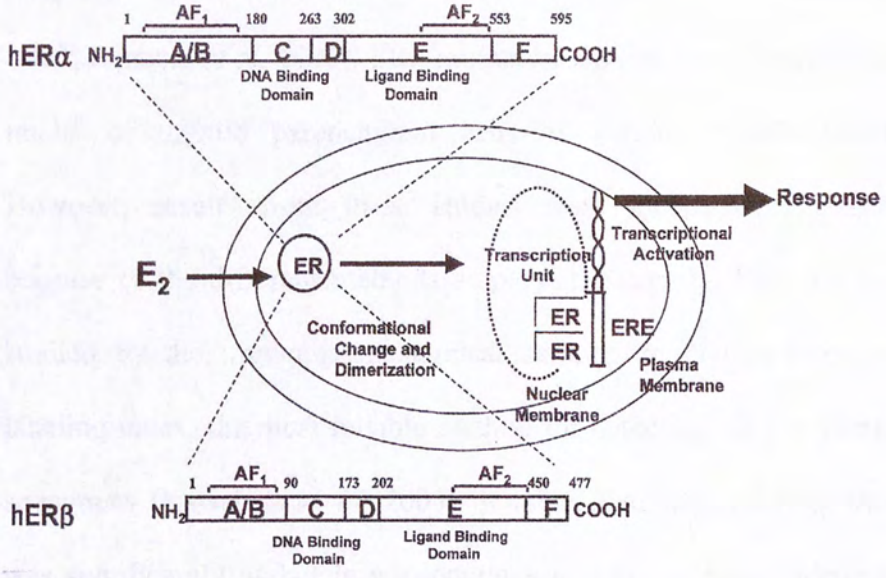


Figure 4. The ERα and ERβ are activated and dimerized to form a transcription unit at an estrogen response element (ERE) in the promoter region of an estrogen responsive gene. The expression of ERα or ERβ target gene is subsequently induced (Handerson 2003).

1.2.4. Estrogen receptor and thyroid cancer

The presence of ERα has previously been examined in human thyroid neoplasms by a number of investigators (Dalle *et al.* 1998; Hiasa *et al,* 1993; Bonacci *et al.* 1996). ERα immunoreactivity was detected in the nuclei of thyroid parenchymal cells of various thyroid lesions. However, results from these studies were inconsistent, possibly because of the different methods employed. Recently, ERα has been studied by the immunohistochemical staining in combination with labeling index, the most reliable method for detecting ERα in clinical specimens (Kawabata *et al.* 2003). It found that ERα labeling index was significantly higher in adenomatous goiters, follicular adenomas and thyroid carcinomas than in normal thyroid glands. In the papillary carcinoma, ERα labeling index was significantly higher in premenopausal women than in postmenopausal women and men of various ages. In contrast, there was no significant correlation between ERβ labeling index and patient age, menopausal status, gender, and tumors, histological type. The higher level of ERα labeling index may be involved in the activation of tumorigenesis of papillary carcinoma in premenopausal female.

1.3 THE ROLE OF APOPTOSIS

Apoptosis is a normal physiological process that regulates cell populations and maintains tissue homeostasis (Wyllie et al., 1980). It is an active process of self destruction that requires the activation of a genetic program that may lead to the change in cell morphology, DNA fragmentation, and protein cross linking. Apoptosis can be triggered in several ways and it involves many cellular functions (Arscott and Baker, 1998). The apoptotic mechanism provides protection of cells from the possible consequences of uncontrolled cell proliferation.

1.3.1 Bcl-family protein

The Bcl-2 family of proteins has been demonstrated to play a key role in the mechanism of apoptosis. There are three classes of proteins that play crucial roles in this complex process: the BH3 domain-only proteins, the pro-apoptotic proteins, and the anti-apoptotic proteins. Bid and Bad, the BH3 domain-only proteins, act as triggers to stimulate the multidomain pro-apoptotic proteins Bax and Bak (Wei *et al.*, 2001). Bcl-2 and Bcl-xL, by sequestering BH3 domain-only proteins, prevent apoptosis (Cheng *et al.*, 2001, Yang *et al.*, 1995).

The Bcl-family gene is widely accepted as regulators of cell death (Minn *et al.*, 1998 and Chao *et al.*, 1998). The anti-apoptotic and pro-

apoptotic genes are considered dominant regulators of apoptosis. Bcl-2 and Bcl-xL confer a negative control in the pathway of apoptosis. In contrast, Bax and Bak promote cell death by competing with Bcl-2 either down-regulation of pro-apoptotic genes or up-regulation of anti-apoptotic genes and thus suppress the growth of tumor cells. Thus, heterodimerization between members of the Bcl-2 family of proteins is a key to the regulation of cell death (Yin *et al.*, 1994).

1.3.2 Bcl-family protein and cancer

The Bcl-family proteins have been widely studied in different kinds of cancer research. For instance, Bcl-xL overexpression was found in hepatocellular carcinoma specimens in 21 of 33 patients (63.6%) and the overall survival rates of the group was definitely poor. These results showed Bcl-xL in HCC specimens, suggesting that Bcl-xL was a significant prognostic factor for disease progression in human HCC. (Watanabe *et al* 2004) In another study, the immunohistochemistry of apoptosis-related proteins in the mucosal biopsy of bladder cancers were analyzed after treatment of chemoradiotherapy (CRT). The Bax/Bcl-2 rate showed a significant association with the clinical response rate. The result of this study suggested that the combined assessment of Bcl-2 and Bax protein expression may be used to predict a clinical response to CRT based on the Bax/Bcl-2 ratio determined before therapy. (Hiroaki *et al*

2004) Therefore, the expression of Bcl-family proteins is critical in determining susceptibility to the abnormal cell growth.

1.3.3 Estrogen and Bcl-family protein

The protective role and the growth stimulatory effect of estrogen have been intensively studied in various ER expressed cells. Estrogen protects neuronal cells and cardiac myocytes from apoptosis by regulation of Bcl-family protein level (Christoph *et al.*, 2001 and Pelzer *et al.*, 2000). It has been demonstrated that estrogen stimulates cell growth in breast and ovary cancer cells by up-regulating Bcl-2 expression (Wang *et al.*, 1995 and Bu *et al.*, 1997). In the results of these studies, the growth stimulatory effect of estrogen in cancer cells may be involved with regulation of bcl-2 family proteins expression. Recently, a study shows that E2 also has a growth-stimulatory role in thyroid cancer cells, similar to the role of E2 in other ER-expressed cells (Manole *et al.*, 2000). However, the effect of estrogen on the expression of Bcl-family proteins has not been investigated in the thyroid cancer cells.

1.4 OBJECTIVES

In this study, we investigated the effect of estrogen (estradiol, E2) on the expression of Bcl-2 family at both member messenger ribonucleic acid (mRNA) and protein levels in the papillary human thyroid carcinoma cell line KAT5, which has a very low Bcl-2 protein expression level (Wang *et al.* 2001). The expression of ER α and β protein levels in E2 treated KAT 5 cell was studied. We also examined the stimulatory effect of the E2 and compared it with the effect of testosterone, a male hormone, in the KAT5 by MTT assay. In addition, the expression of Bcl-2 family member proteins was investigated in the KAT5 cells that were treated with the two sexual hormones. Using Western blot analysis, we investigated the anti-apoptotic potential (Bcl-xL/Bax ratio) that is believed to be crucial for the regulation of apoptosis in thyroid cancer. Bcl-xL, like Bcl-2, protects cells from a wide variety of apoptotic stimuli. In contrast, Bax, Bcl-2 like protein, binds to and antagonizes the protective effect of Bcl-2 and Bcl-xL, and renders cells more sensitive to death stimuli. In this sense, the ratio of expression of Bcl-xL to Bax appears to determine cell fate in an adverse microenvironment. Moreover, the expression of Bcl-xL and Bax mRNA was studied to see whether mRNA data consistent with the level of protein expression. The results indicate that the action of on the growth of KAT5 cells may be associated with up-regulation of Bcl-xL gene at both mRNA and protein levels.

CHAPTER TWO: GENERAL MATERIALS AND METHODS

2.1 Materials

2.1.1 Culture Media and treatment Agent

Material	Supplier
Charcoal treated FBS	HyClone (Logan,Utah)
RPMI 1640 without phenol red	GIBCO-BRL (Carlsbad, CA)
Penicillin/ampicillin/streptomycin	GIBCO-BRL (Carlsbad, CA)
Beta-estradiol	Sigma (St. Louis, MO)
Testosterone	Sigma (St. Louis, MO)
Trypsin-EDTA	GIBCO-BRL (Carlsbad, CA)
Tamoxifen	Sigma (St. Louis, MO)

2.1.2 Reagents for Western Blot

Material	Supplier
PMSF	Sigma (St. Louis, MO)
Sodium Orthovanadate	Sigma (St. Louis, MO)
Aprotinine	Sigma (St. Louis, MO)
Acrylamide/Bis-acrylamide	Boehringer Mannhein (Mannheim, Germany)
Dithiothreitol (DTT)	Sigma (St. Louis, MO)
Nonidet P-40	Amersham(Buckinghamshire.UK)
Proteins molecular weight marker Full range rainbow marker	Amersham(Buckinghamshire.UK)
ECL membrane	Amersham(Buckinghamshire.UK)
Hyper ECL Film	Amersham(Buckinghamshire.UK)
SDS	Amersham(Buckinghamshire.UK)

2.1.3 Anitbodies

Antibody	Supplier
Mouse monoclonal anti-Bcl-xL IgG	Santa Cruz (Santa Cruz, CA)
Mouse monoclonal anti-Bax IgG	Santa Cruz (Santa Cruz, CA)
Rabbit polyclonal anti-ERα IgG	Santa Cruz (Santa Cruz, CA)
Rat monoclonal anti-β tubulin IgG	Abcam (Cambridge, UK)
Goat anti-mouse HRP-IgG	Santa Cruz (Santa Cruz, CA)
Goat anti-rabbit HRP-IgG	Santa Cruz (Santa Cruz, CA)
Rabbit anti-rat HRP-IgG	Santa Cruz (Santa Cruz, CA)

2.1.4 Materials for RT-PCR

Material	Supplier
Agarose	Sigma (St. Louis, MO)
Blue/orange 6x loading dye	Promega (Madison, WI)
Taq DNA polymerase	Promega (Madison, WI)
DNA ladder Markers (1kb, 100bp)	Promega (Madison, WI)
AMV reverse transcriptase	Promega (Madison, WI)

2.1.5 Kits

Product	Supplier
RNeasy Mini Kit	Qiagen (Cologne, Germany)
Reverse Transcription system	Promega (Madison, WI)
Protein DC Assay	Bio-Rad (Hercules, CA)
ECL Western Blot Detection Kit	Amersham(Buckinghamshire,UK)

2.1.6 Instrumentation

Instrument	Supplier
Eppendorf centrifuge 5415D	Eppendorf (Hamburg, Germany)
Eppendorf centrifuge 5417R	Eppendorf (Hamburg, Germany)
Mini orbital shaker	Bibby (Staffordshire, UK)
RNA/DNA calculator	Amersham(Buckinghamshire,UK)
UV gel documentation System	UVItec limited (Cambridge, UK)
GeneAmp PCR system 9600	Perkin Elmer (Foster City, CA)
Mini trans-Blot cell	Bio-Rad (Hercules, CA)
Model GS-700 imaging densitometer	Bio-Rad (Hercules, CA)
Molecular Devices microplate reader	Tecan Spectra (Princeton, NJ)

2.2 Methods

2.2.1 Cell Culture and Treatment

The monolayer of the human thyroid papillary carcinoma cells KAT5 was grown in RPMI 1640 medium supplemented with 10 % FBS, 100 U/mL penicillin and 100ug/mL streptomycin. At 70 %-80 % confluence of KAT5 cells, the cell was trypsinized by 1 % EDTA-Trypsin. After centrifugation and aspiration of the supernatant, the cells were plated at 2 x 10^6 cells/plate in 12 ml phenol red free RPMI 1640 supplemented with 0.5-1 % charcoal-stripped FBS in 100 x 15 mm plates. The cells were then grown at 37 °C in 5 % CO_2 for 48 hours before stimulation with beta estradiol or testosterone. After 48-hour incubation, beta estradiol and testosterone at various concentrations (both at 10^{-11}, 10^{-9}, and 10^{-7} mol/L), and vehicle (0.02 % EtOH) were added to the KAT5 cells in fresh medium, and the cell cultures were incubated at 37 °C in 5 % CO_2 for 1 and 2 days.

2.2.2 MTT Assay

Cell viability was assessed by the mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. KAT5 cells were incubated with 10^{-11}, 10^{-9}, and 10^{-7}mol/L of either beta-estradiol or testosterone in 96 well-plates before the addition of MTT. After the removal of conditioned medium, the cells were incubated with MTT in medium (0.5mg/ml) for 3 hours. The purple formazan product was solubilised in 0.04N HCl-isopropanol. The absorbance of each sample at 570nm was measured using a Molecular Devices microplate reader. The percentage of living cells was calculated by the following formula: absorbance of the treated well/absorbance of the control well x 100 %.

2.2.3 Western Blot Analysis

2.2.3.1 Protein Extraction

Cells were rinsed with PBS and scraped off the cell culture plates with lysis buffer containing 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, PMSF, aprotinin, and 0.1 mM sodium orthovanadate. The cells were further disrupted and homogenized by passing through a 21-gauge needle. Having incubated on ice for 1 hour, the cell lysate was centrifuged at 12000 rpm for 10 minutes at 4 °C. The concentration of protein was determined using the Bio-Rad DC Protein assay. The protein samples were kept at –20 °C.

2.2.3.2 SDS-PAGE electrophoresis and protein transfer

The protein sample was mixed with 6X sample buffer and denatured by boiling at 95 °C for 5 minutes. 30 micrograms of protein per lane were loaded and separated on a 10-12 % SDS-polyacrylamide gel. Electrophoresis was carried out at room temperature at 100 V for 1.5 hours. The gel was calibrated with Rainbow markers. After electrophoresis, the protein was electrotransferred onto a Hybond ECL Nitrocellulose membrane using a Bio-Rad Mini Trans-Blot Cell at 4 °C, 100 V for 1 hour.

2.2.3.3 Immunoblotting analysis

After protein transfer, the membrane was blocked by 5 % nonfat dry milk in 1 x TBST buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 % Tween-20) for 1 hour. They were then incubated with a relevant primary antibody overnight at cold room, washed three times with TBST buffer and then incubated with a correspondent HRP-conjugated secondary antibody for two hours at room temperature. The name and the condition of the antibodies used were listed in Table 2.

Table 2. Antibodies used for immunoblotting analysis

Primary Antibody	**Dilution**	**Second Antibody**	**Dilution**
Bcl-xL mouse monoclonal IgG	1:250	Goat anti-mouse HRP-IgG	1:1000
Bax mouse monoclonal IgG	1:500	Goat anti-mouse HRP-IgG	1:1000
ERα rabbit polyclonal IgG	1:500	Goat anti-rabbit HRP-IgG	1:1000
Tubulin rat monoclonal IgG	1:1000	Rabbit anti-rat HRP-IgG	1:1000

After the membrane was washed for three times by TBST buffer, an enhanced chemiluminescene (ECL) system was applied on the membrane. The membrane was exposed to ECL films from 30 seconds to 5 minutes, depending on the intensity of the targeted signal. Quantitation of the protein was performed with GSP-700 scanner with Quantity One image software (Hercules, CA).

2.2.4 RNA extraction and RT-PCR

2.2.4.1 RNA Extraction

Total RNA was isolated with the RNeasy Kit from QIAGEN and performed according to the manufacturer's instruction. Briefly, the cell pellet was washed with PBS once, and then was disrupted by Buffer RLT with β-ME. After lysate was homogenized to shear genomic DNA and reduce viscosity of lysate with QIA shredder column, 1 volume of 70 % ethanol was added to adjust the binding condition. Subsequently, the mixture was applied to RNeasy mini spin column for adsorption of RNA to a membrane. Contaminant was removed by simple wash spins (buffers RW1 and RPE). The total ready-use RNA was eluted with RNase-free water and stored at –70 °C. The concentration of the RNA sample was determined using a DNA/RNA reader.

2.2.4.2 cDNA synthesis

The 1μg of total RNA was used to synthesize cDNA in a reaction mixture containing 15U AMV reverse transcriptase. The reaction was carried out in a 0.5ml eppendorf tube at 42°C for 45 minutes. After reaction, the tube was heated at 99°C for 5 minutes to inactivate the enzyme and thus stop the reaction.

Reverse transcription 10X Buffer	2	μL
DNTP mixture (10mM)	2	μL
MgCl2 (25mM)	4	μL
RNasin Ribonuclease Inhibitor	0.5	μL
AMV reverse Transcriptase (20u/μL)	0.75	μL
Oligo $(dT)_{15}$	0.5	μg
Total RNA template	1	μg
Nuclease-free water to final volume	20	μL

2.2.4.3 Polymerase Chain Reaction (PCR)

The synthesized cDNA was used for PCR to amplify the target gene. All of the reactions in this study were carried out under the same condition. The reaction was set up as follows:

10x PCR buffer	2	µL
dNTP (10mM)	2	µL
$MgCl_2$(25mM)	1	µL
Primer (Forward)	0.5	µL
Primer (Reverse)	0.5	µL
Nuclease-free H_2O	12.5	µL
Taq (5U/µL)	0.5	µL
Template	1	µL
Total Volume	20	µL

PCR amplification was performed using a programmable thermal cycler. Reactions were carried out at 94 °C for 2 min; 30 cycles of 94 °C for 35 sec, 58.6 °C for Bcl-xL or 49 °C for Bax or 62 °C for β actin for 35 sec, 72 °C for 35sec; and then 72 °C for 10 minutes and 4 °C to terminate the reaction. Electrophoresis of 5µl of each PCR reaction was performed in a 1 % agarose gel. The primers used were detailed in Table 3.

Table 3. Sequences and positions of synthetic oligomers used in this study:

Primer	Sequence	Orientation
Baxa F	5'-TTT TCT GAC GGC A AC TTC –3'	Sense
Baxa R	5'-GCG AGG CGG TGA GCA CTC C-3'	Antisense
Bcl-xL F	5'-CAT CAC CCC AGG GAC AGC ATA TC-3'	Sense
Bcl-xL R	5'-TGG TCA TTT CCG ACT GAA GAG TG-3'	Antisense
β actin F	5'-GCA TTT GCG GTG GAC GAT GGA GG-3'	Sense
β actin R	5'-GGT CAC CCA CAC TGT GCC CAT CTA-3'	Antisense

2.2.5 Statistical Analysis

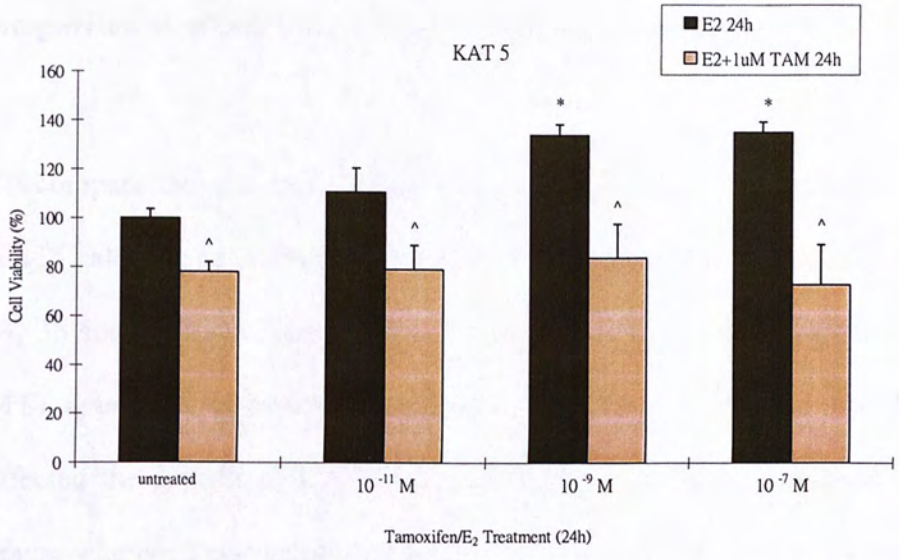
The statistical analysis was performed using a SPSS 8.0 for Windows (SPSS, Inc., Chicago, IL). ANOVA for repeated measurements was applied to compare cell viability MTT assay and the level of target proteins in the cell treated with different concentrations of E2 and testosterone.

CHAPTER THREE: RESULTS

3.1 Effect of E_2 and tamoxifen on proliferation

The viability percentage of KAT5 cells treated with E2 was determined by the MTT assay. The viability percentage was calculated as dividing the absorbance 570nm of wells with E2 treatment by the absorbance 570nm of the untreated cell and multiplied by 100. The data was presented as the mean of living cell percentage in four individual experiments on y-axis versus the different E2 treatments on x-axis. As shown in Figure 5A, E2 at concentrations of 10^{-9} and 10^{-7} M caused a significant increase in cell proliferation at 24-hour incubation compared with untreated cells and 10^{-11} M E2 (*P<0.05, n=4), and the increase reached a plateau after the concentration of E2 reached 10^{-7} M. In 48-hour culture (Figure 5B), the increase was reduced after the concentration of E2 reached 10^{-7} M. The highest proliferative effect of E2 was observed at 10^{-9} M in 24 and 48 hour incubations. The growth of cells was statistically decreased in the cells treated with tamoxifen plus E2 compared with the cells received no tamoxifen treatment (^P<0.05, n=4). The cotreatment with E_2 plus tamoxifen (10^{-6} M) could attenuate the effect of E2 in KAT5 cells to the level of untreated samples in both 24-and 48-hour groups (Figure 5A and 5B).

5A



5B

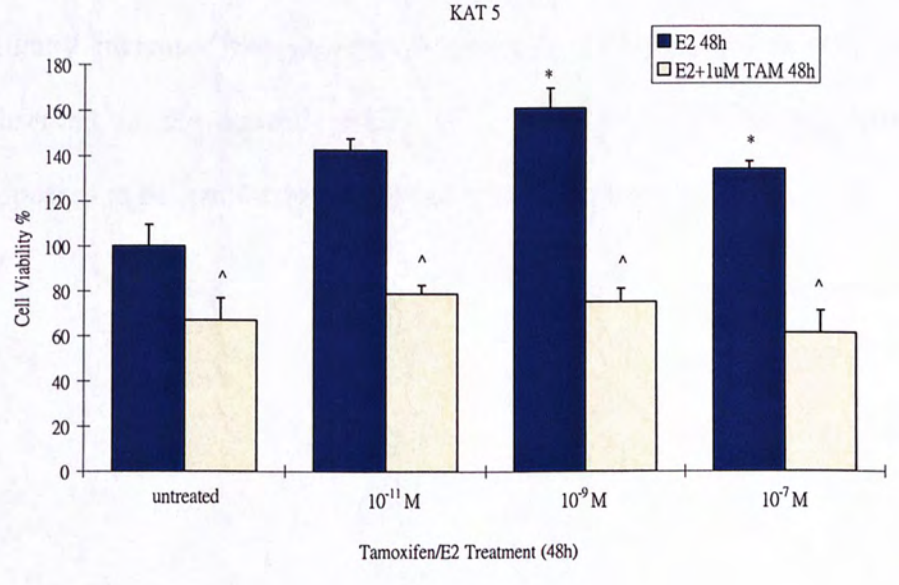


Figure 5A and B. The proliferation of KAT5 cells determined by MTT assay. A significant increase was observed (*versus* untreated cells, *P<0.01, n=4) in response to E2 in both 24- and 48-hour incubations. Such an increase was markedly reduced after the addition of tamoxifen (versus each treated cells, ^P<0.05, n=4). Data are the mean ± S.D. of 4 separate experiments.

3.2 Comparison of effects of E_2 and Testosterone on proliferation

To compare the effects of E2 and Testosterone on cell proliferation in KAT5 cells, the cells were treated with 1nM of E2 or Testosterone for 12, 24, 36 and 48 hours. The absorbance of 570nm for formazan product in MTT assay was measured as previously described. Treatment with E2 affected the growth of KAT5 and reached the maximum growth in 24 hours, whereas Testosterone treatment for 24 hours resulted in a decrease of the growth in KAT5 (Figure 6). The growth of KAT5 was plateau after 36 hours treatment with E2. In contrast, treatment with testosterone slightly increased the growth of KAT5 in 36 hours, but it was also observed in the control group. Therefore, stimulatory growth effect appeared to be specific to E2 and the effect was dose dependent.

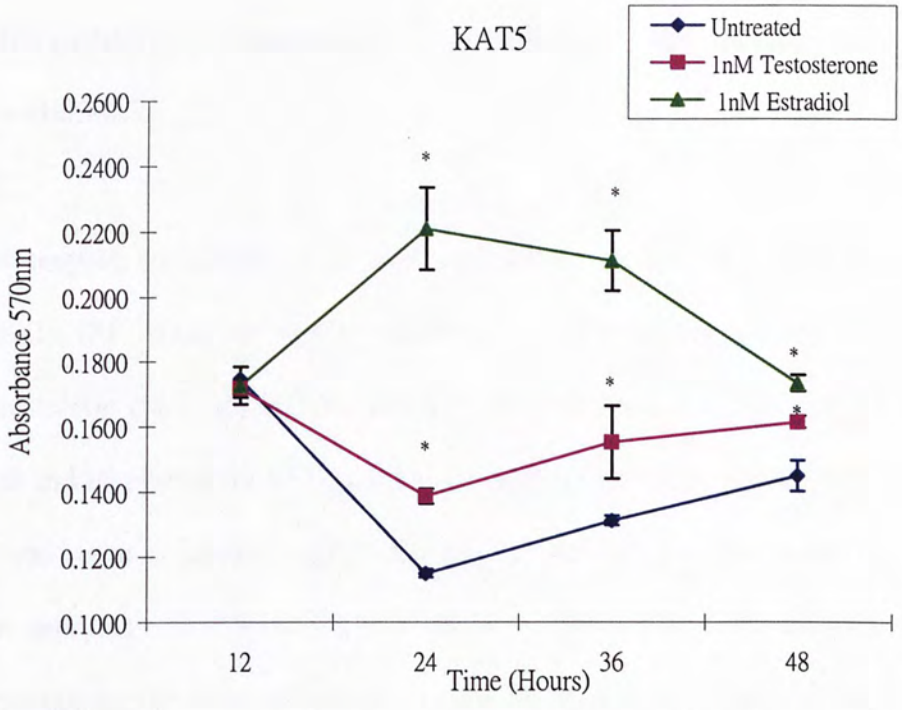


Figure 6. Effect of E2 and testosterone on cell proliferation. To compare the effect of E2 and testosterone on the growth of KAT5 cells, the cells were treated with 1 nM of E2 and testosterone for 12, 24, 36 and 48 hours. The absorbance of 570 nm was analyzed following the addition of MTT substrate after different incubation periods indicated. Data are shown as the mean of four individual experiments and presented as the mean ± S.D. (*P<0.05 *versus* untreated group at specific time point, n=4).

3.3 Differential Bcl-xL expression in response to E_2 and testosterone stimulation.

To compare the effects of E2 and testosterone on the expression of Bcl-xL (31 kDa) in KAT5, different concentrations of E2 or testosterone (10^{-11} M, 10^{-9} M, and 10^{-7} M) were added to the KAT5 cells and incubated for 24 hours. Mouse monoclonal antibody for Bcl-xL and rat monoclonal antibody for tubulin were used in the Western blot analysis. The Western blot analysis showed that E2 clearly increased in the level of Bcl-xL in a dose-dependent manner. The maximal effect was found at the concentration of 10^{-7} M (Figure 7). Unlike E2, in KAT5 cells stimulated with testosterone, the level of Bcl-xL remained constant for three concentrations of testosterone tested, which were not different from untreated cells. Tubulin (52 kDa) expression was used to check the loading size into each lane.

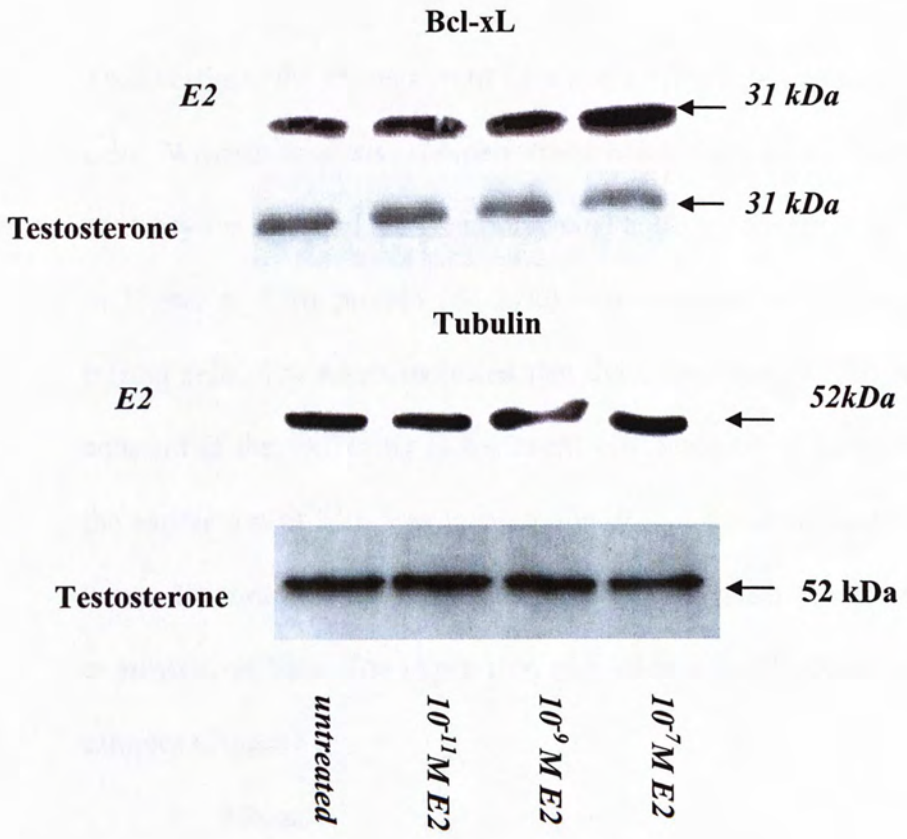


Figure 7. Expression of Bcl-xL protein. The level of Bcl-xL (31 kDa) protein in KAT5 treated with E2 and testosterone was investigated by Western blot analysis. The protein amount was normalized by tubulin protein (52 kDa). At least three individual experiments were performed.

3.4 ERα and ERβ expression in response to E_2 stimulation

To investigate the expression of ERα and ERβ in E2-stimulated KAT5 cells, Western analysis was performed using the mouse monoclonal antibody for ERα and the goat polyclonal antibody for ERβ. As shown in Figure 8, ERα protein (68 kDa) was observed in untreated and treated cells. The result indicated that the expression of ERα remains constant in the increasing E_2 treatment concentration in 24 hours, and the expression of ERα was increased in dose dependent manner in 48 hours. In contrast, the expression of ERβ was much lower than the expression of ERα. The expression of ERβ was hardly detected in all samples (Figure 8).

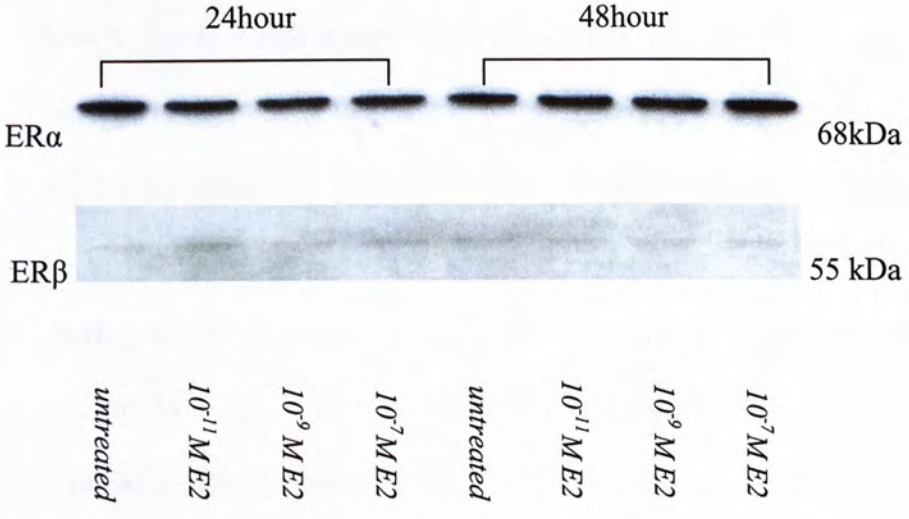


Figure 8. Effect of E2 treatment on the expression of ERα and ERβ proteins in KAT5 cells. The protein level was investigated by Western blot analysis. The ERα and ERβ were expressed at the size of 68 kDa and 55 kDa.

3.5 Bcl-xL and Bax protein expression in response to E_2 stimulation

Western blot analysis was used to determine relative levels of Bcl-xL and Bax protein in each of the three E2 treatment groups, and tubulin was used to monitor sample loading (Figure 9A). Cells treated with E2 for 24 hours showed a clear increasing Bcl-xL protein level, compared with that in the control group. No difference was observed in the expression of Bcl-xL protein in 48-hour incubation. The expression of Bax protein appeared unchanged in all samples treated with E2 at both time points. The ratio of anti-apoptotic Bcl-xL to pro-apoptotic Bax was used as a relative indicator of anti-apoptotic potential (Adams and Cory 2001). This study determined this ratio by measuring the density of the target protein bands. Cells treated with E2 showed (Figure 9B) a significant increase in the ratio of Bcl-xL to Bax compared with that in control group in 24-hour incubation. There was no significant difference in ratio of Bcl-xL to Bax between untreated cells and cells treated with E2 in 48-hour incubation. The increased anti-apoptotic potential in the cells treated with E2 for 24 hours was consistent with the result of the E2 growth-stimulated effect obtained by MTT analysis.

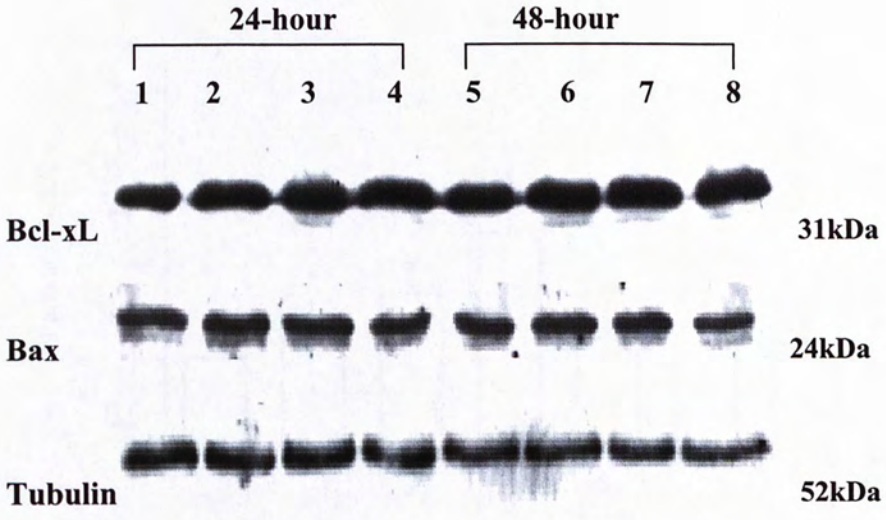


Figure. 9A Effect of E2 on the expression of Bcl-xL (31 kDa), Bax (24 kDa) and tubulin (52 kDa) proteins in KAT5. Lanes 1-4: 24-hour treatment. Lane 1: control; Lane 2: 10^{-11}M; Lane 3 : 10^{-9}M; Lane 4 : 10^{-7}M. Lanes 5-8: 48-hour treatment. Lane 5: control; Lane 6: 10^{-11}M; Lane 7: 10^{-9}M; Lane 8 : 10^{-7}M.

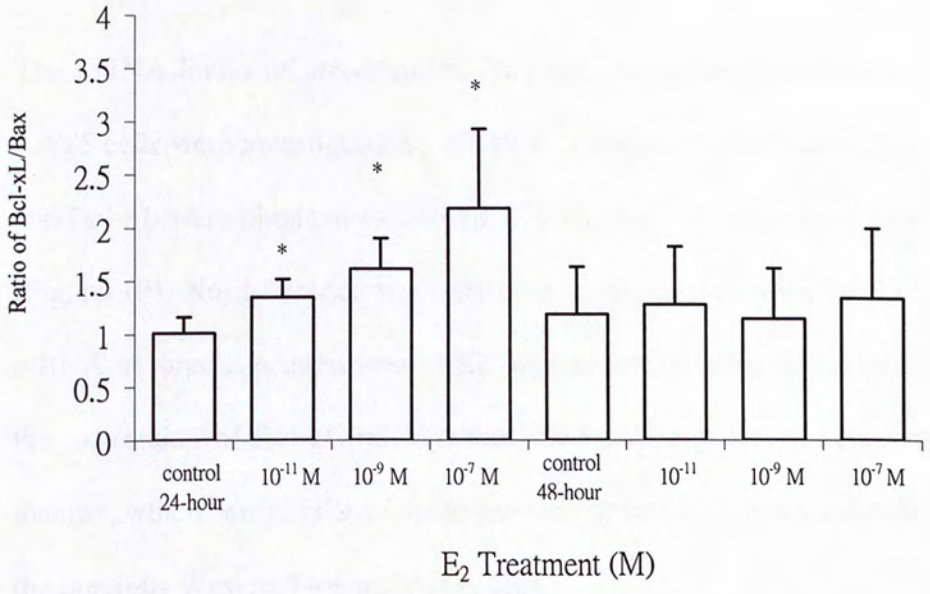


Figure 9B. The semi-quantitative analysis of the Western blots of anti-apoptotic potential (Bcl-xL/Bax ratio). The Bcl-xL and Bax protein bands were scanned. Their densities were determined by and the ratio of Bcl-xL and Bax was calculated. Significant difference was determined using one-way ANOVA(* $P<0.05$ *versus* untreated for 24-hour group). At least three separated experiments were performed.

3.6 Bcl-xL and Bax mRNA expression in response to E_2 stimulation

The mRNA levels of pro-apoptotic Bax and anti-apoptotic Bcl-xL in KAT5 cells were investigated by RT-PCR. PCR products of actin, Bax and Bcl-xL were obtained as 649 bp, 256 bp, and 367 bp respectively (Figure 10). No difference was observed in expression level of Bax mRNA in three concentrations of E2 -treated KAT5 cells. In contrast, the expression of Bcl-xL mRNA was increased in a dose-dependent manner, which was similar to the expression of Bcl-xL protein result in the previous Western blot analysis results.

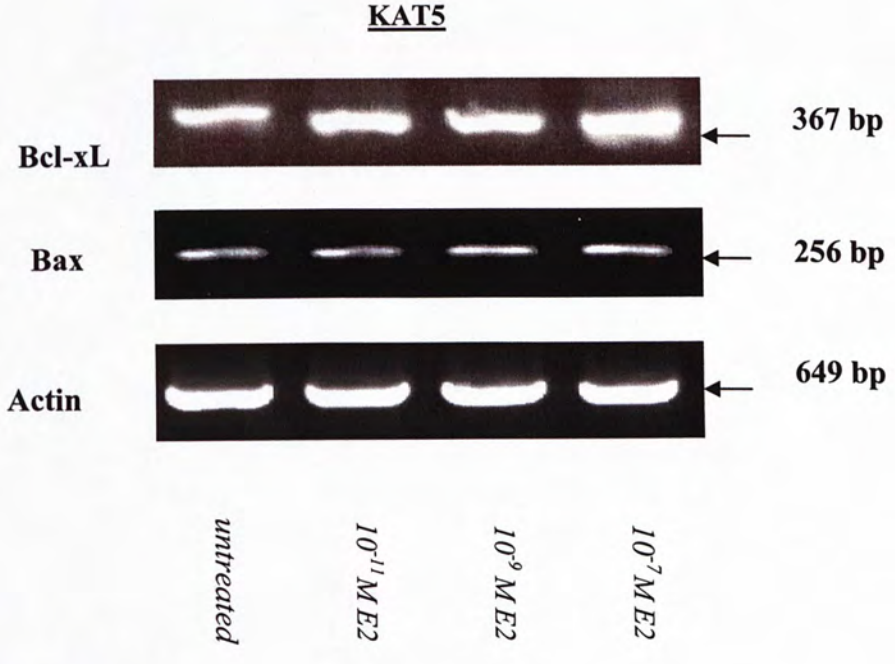


Figure 10. Effects of E2 on the levels of Bax and Bcl-xL mRNA. To investigate the mechanism of E2 in the stimulation of growth, the regulation of

Bax and Bcl-xL was examined using RT-PCR in KAT5 cells. The cells were treated with E2 for 24 hours. As expected the sizes of PCR products for Bcl-xL and Bax were 367 bp and 256 bp respectively. At least three individual experiments were performed.

CHAPTER FOUR: DISCUSSION

Tissue homeostasis is regulated in part by factors that control the appropriate balance between cell proliferation and cell death. If tumors are to arise they must successfully bypass the cell-killing program that is activated in response to genetic and environmental factors (Fagin 1998). E2 has been studied as one of the factors that influence tumorigenesis in various ER expressed cells (Gao *et al.* 2002, Franke *et al.* 2002, and Choi *et al.* 2000). In breast cancer cells, the E2 in a concentration of 10^{-6} M induced proliferation in ER+ breast carcinoma cells. In addition, treatment with exogenous E2 (10^{-8}-10^{-6} M) resulted in a growth stimulation of tumorigenic ovarian cell line by up-regulating the anti-apoptotic Bcl2 mRNA and protein. In uterine leiomyoma cells, E2 (10 ng/ml) treatment significantly decreased the tumor suppressor p53 protein level to regulate the growth of the cancer cells. Thyroid tumors highly express ER (Inoue *et al.*1993, Yane *et al.*1994, and Clark *et al.*1985) in the same way as estrogen-dependent carcinomas such as those of ER expressed breast, ovarian and uterine tissues. Recently, the growth-promoting effect of E2 in benign and malignant thyroid cells has been confirmed and extended to its influence in the regulation of cell cycle by inducing cyclin D1 protein levels (Manole *et al.*2001). However, the mechanism how E2 affects the programmed cell death has not yet been investigated in thyroid cancer cells.

The present study first tested whether sex hormones had any effect on the proliferation and growth of human thyroid cancer cell, KAT5 papillary thyroid carcinoma cell. The result demonstrated the specificity of E2 on growth promotion in KAT5 that tamoxifen reversed the effect of E2 on the cell, suggesting that the proliferation of KAT5 cells by E2 was mediated through its specific receptors. Treatments of E2 (10^{-11} M to 10^{-9} M) increased the growth of KAT5 cells in both 24- and 48-hour incubation. But the stimulatory growth effect of E2 was not significant at 10^{-11}M E2 treatment at 24-hour incubation and it reached a plateau at the concentration of 10^{-7} M in 24-hour incubation. In addition, the growth effect of E2 was decreased at the concentration of 10^{-7} M when the incubation time was 48-hour. The similar result was also demonstrated by Yu *et al.* 1996. Yu et al reported that E2 at low concentrations has no stimulatory effect on the growth of human thyroid carcinoma cell lines in 24–hour incubation. However, these authors reported a marked reduction in the proliferation of the thyroid cancer cell lines exposed to the E2 concentration at 10^{-6} M. Taking together the data from the present study and that of Yu *et al.*, it implied that the growth effect of E2 was in a dose dependent manner.

The effects of E2 and testosterone on cell proliferation in KAT5 cells were compared by treating the cells with 1nM of E2 and testosterone for 12, 24, 36 and 48 hours. The result showed that E2 increased the cell proliferation and that its effect reached the maximal effect at the 24-hour point of treatment. In

contrast, testosterone markedly decreased the cell proliferation in 24-hour incubation, though the reduction of the cell proliferation was also found in the control group. Thus, the result suggested that E2 and testosterone differentially affected the proliferation of the KAT5 cells. Also, the Bcl-xL expression in KAT5 cells was determined by Western blot after treatments of E2 and testosterone for 24 hours. E2 clearly increased the level of Bcl-xL in a dose-dependent manner. Unlike E2, testosterone did not cause any difference between treated and untreated KAT5 cells in the level of Bcl-xL expression. The differential effects of E2 and testosterone on the proliferation of KAT5 cells and the expression of Bcl-xL level in KAT5 cells provided a good laboratory evidence, that sex steroids may be one of the factors underlying the gender bias in thyroid cancer incidence. Thus it may explain the epidemiological observation that thyroid cancer occurs predominantly in females, especially those in their reproductive age.

The effect of E2 is believed to be closely associated with its receptors. Two estrogen receptor isoforms have been identified. It has subsequently shown that ERα and ERβ proteins can create three possible transactivating factor combinations- ERα homodimers, ERβ homodimers and ERα-ERβ heterodimers in several experimental systems (Pace et al. 1997, Petterson et al. 1997). These dimers bind to their specific estrogen response elements (EREs) that initiate transcription of specific genes. Therefore, differential gene expression in a cell will be determined by the ER expression and its promoters.

In present study, the results showed that ERα expression was higher than ERβ expression in KAT5 cells treated with the three different E2 concentrations at both 24- and 48-hour points of time. The ERα expression was slightly increased in the cell treated with E2 for 48 hours however it remained unchanged in the cell treated for 24 hours. In contrast, the ERβ expression remained unchanged in all conditions at all points of time. A similar result was also found by Manole *et al.* 2001. They found that the level of ERα had a maximal expression at 48-hour in different E2 concentrations, and the ERβ expression remained constant in the human thyroid carcinoma cell line. In another study by Choi *et al.* 2000, the expression levels of ERα were enhanced in ovary carcinoma cells when compared with those in normal ovaries, whereas ERβ levels were significantly decreased in ovarian tumors. The result suggested that differential expression of ERα and ERβ in the tumors may be altered in response to estrogen treatment and the expression of ERα may be specific in response to E2 in the KAT5 tumor cells.

Among pro-apoptotic and anti-apoptotic genes in Bcl-2 family, the expression of Bax and Bcl-2 genes has been studied as the dominant regulators for apoptosis. The ratio of Bcl-2 to Bax was important in determining susceptibility to apoptosis (Chao *et a.l* 1998). The present study has demonstrated that Bax and Bcl-xL, a homolog of Bcl-2, were expressed at both mRNA and protein levels in KAT5 cells. No difference was observed in the expression level of Bax mRNA among the different E2 treated-KAT5 cells. In contrast, treatment with E2 resulted in an obvious increase of Bcl-xL

mRNA level. These findings were in agreement with a previous report where estrogen up-regulated anti-apoptotic Bcl-2 gene, whereas Bax level was not affected by E2 in breast cancer cells (Perillo *et al* 2000). The up-regulation of Bcl-xL by E2 suggested that E2 affected the survival of KAT5 cells through a pathway involved with Bcl-xL, which was known to be a regulator of apoptosis (Pike 1999, Leung *et al.* 1999). In the study by Leung *et al.* (1999), it showed that estrogen down-regulated anti-apoptotic Bcl-xL mRNA and up-regulated anti-apoptotic Bcl-2 mRNA and protein in a dose-dependent manner in breast cancer cells, suggesting that members of bcl-2 family may be differently regulated in different types of cancer cells. In parallel with mRNA level, E2 caused a significant up-regulation of Bcl-xL protein level, whereas no difference was observed in Bax protein level. KAT5 cells treated with E2 for 24 hours showed a significant up-regulation of the ratio of Bcl-xL to Bax compared with that in control cells.

However, the increased ratio of Bcl-xL to Bax by E2 treatment was reduced as there was no significant difference in this ratio between untreated cell and cells treated with E2 while the growth of KAT5 cell still increased in dose-dependent manner in range of 10^{-11}M to 10^{-9}M of E2 treatment at 48-hour incubation. E2 can trigger multiple signaling pathways, such as MAP kinases, PI3-kinases, Protein Kinase A (PKA) and cyclin D1 (Kelly *et al.* 1999, Manole *et al.* 2001, Sun *et al.* 2001). In addition, the ligand-bound estrogen receptor can bind to estrogen responsive element (ERE) in the promoter region of estrogen-responsive genes and activate these genes, such as c-myc, c-fos, Ap-1,

FasL (Mor *et al.* 2000, Kelly *et al.* 1999, Santen *et al.* 2001) and Bcl-2. For example, changes in protein profile during the adaptation of breast cancer cells to long-term estrogen deprivation have been demonstrated previously, involving up-regulation and activation of several critical regulatory proteins, such as c-Myc, c-Myb, MAP kinase, E2F1, and Ras (Chen *et al.* 2000, Cobb *et al.* 1999, Prendergast *et al.* 1999). These proteins can mediate the action of hormones and growth factors by exerting stimulating proliferation on the one hand or inducing apoptosis on the other, depending on the conditions (Bacus *et al.* 2001, Evan *et al.* 1998). In this study, estrogen did affect cell proliferation in papillary human thyroid carcinoma cells, KAT5 cells, and the mechanism of estrogen in regulation of anti-apoptotic pathway may be associated with up-regulation of Bcl-xL gene.

CHAPTER FIVE: CONCLUSION

Taken together the data from the present study and that of other previous studies, it becomes clear that the effect of E_2 on growth of thyroid carcinoma cells may involve more than one pathway. The present study demonstrates that E_2 promote papillary thyroid cell growth in part through the apoptotic pathway by up-regulating Bcl-xL. It may provide the insights into the molecular mechanism underlying the epidemiological data that show a prevalence of thyroid carcinomas 2- 3 folds more in females than in males. From the therapeutic point of view, the finding that the enhancement of anti-apoptotic signaling by E_2 may be significant in the prevention or treatment of thyroid tumors, because anti-estrogen or anti-ER medication can inhibit the growth of thyroid cancer cells via a pathway pf promoting apoptosis.

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