Apoptotic Effects of Iodine in Thyroid Cancer Cells

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ABSTRACT

Thyroid cancer is the most common endocrine malignancy and exhibits the full range of malignant behaviors from the relatively indolent occult differentiated thyroid cancer to uniformly aggressive and lethal anaplastic thyroid cancer. Iodine is a well known key element in thyroid normal function maintenance and thyroid cancer development. However, the mechanisms of iodine in thyroid cancer cells development are limited. Recent researches have indicated that iodine could induce cancer cells apoptosis, staying clear from the dysfunction of iodide-specific transportation systems in thyroid cancer cells. Thus, iodine-induced apoptosis may be an effective pathway for iodine to affect thyroid cancer development, but we know little about them.

This reseach firstly investigated iodine-induced apoptotic effects and the underlying mechanism in thyroid cancer cells. Results indicated that apoptosis induced by iodine, especially at high dose of iodine (100 μM), was mitochondrial-mediated, with the loss of mitochondrial membrane potential, Bak up-regulation, caspase 3 activation and cytochrome C release from mitochondria. Iodine treatment decreased the level of mutant p53 including the R273H mutant that possesses anti-apoptotic features while increased the p21 level. The block of p21 significantly prevented iodine-induced apoptosis. High doses of iodine also stimulated the transient activation of the subfamily members of MAPKs (ERK1/2, p38 and JNK1/2). The results showed the three subfamily members of MAPKs all worked as anti-apoptotic factors. Surprisingly, high doses of iodine promoted instead of suppressed the expression of anti-apoptotic protein Bcl-xL expression. The increase

of Bcl-xL was likely to compensate the damage induced by iodine since the inhibition of Bcl-xL accelerated iodine-mediated apoptosis. Collectively, iodine induced mitochondrial-mediated apoptosis in thyroid cancer cells. This apoptotic pathway was involved in the activation of MAPKs pathways, which may subsequently up-regulate p21, Bcl-xL, and down-regulate anti-apoptotic mutant p53 expression. The findings provide solid molecular evidence to explain the epidemiological observation that iodine insufficiency promotes the thyroid tumor development. It may also reveal some novel molecular targets for the treatment of thyroid cancer.

To further explore iodine on the apoptotic effects of chemotherapeutic agents in thyroid cancer, anaplastic thyroid cancer cell line ARO was used. Anaplastic thyroid cancer is lethal because of its rapid progression and poor response to chemotherapy and radioiodine therapy. The study examined the effect of moderate dose of iodine (50 μM) on the apoptosis of ARO cells treated with doxorubicin (Dox) and histone deacetylase inhibitor sodium butyrate (NaB). The cytotoxic effect of either Dox or NaB alone was limited, but co-administration of NaB and Dox (NaB-Dox) significantly increased mitochondrial-mediated apoptosis. The effects of iodine to apoptosis-induced by the two agents were diversified. Iodine reduced the apoptosis induced by Dox or NaB-Dox but promoted apoptosis induced by NaB. To explain this diversifying finding, the experiment found that iodine exaggerated NaB-mediated Bcl-xL down-regulation. In contrast, it reduced the effect of Dox on the decrease of Bcl-xL expression. Further experiments showed that iodine regulated the level of Bcl-xL in ERK- or/and p38-related pathways. The balance between ERK and p38 may determine the iodine-modulated Bcl-xL expression. The high ERK/p38 activity

ratio up-regulated Bcl-xL and enabled the tumor cells to resist chemotherapy, whereas the low ERK/p38 down-regulated Bcl-xL and sensitized the tumor cells to chemotherapy. Taken together, iodine plays a critical role in apoptosis of thyroid cancer cells induced by chemotherapeutic agents. The balance between ERK and p38 may determine cell survival and death through modulating Bcl-xL expression in thyroid cancer cells. The findings provide some new insights into the roles of iodine in chemotherapeutic agents-induced apoptosis in thyroid cancer cells.

To summarize, iodine-induced apoptotic effects on thyroid cancer cells is a key pathway for iodine to influence thyroid cancer development and chemotherapy. Meanwhile MAPKs-related mutant p53, p21 and Bcl-xL expression are critical in deciding thyroid cancer cells survival and death. Moreover, iodine can influence chemotherapeutic agents-induced apoptosis through ERK/p38-mediated Bcl-xL expression.

摘 要

甲狀腺癌是發病率最高的內分泌系統腫瘤，并且其預后隨著甲狀腺癌種類的不同而差異顯著。分化較好的甲狀腺癌預后極好，甚至可以治愈。而未分化甲狀腺癌則預后極差，生存期僅數月。碘作為人體內必需元素之一，與甲狀腺癌的發生密切相關，同时放射性碘（^{131}I）被廣泛應用于甲狀腺癌的診斷和治療。但是目前對于碘在甲狀腺癌的發生、發展以及治療的研究卻非常有限，其機理也不清楚。近來的研究表明，碘即使在碘離子轉運蛋白系統功能存在缺陷的情況下也可以誘導腫瘤小寶凋亡。因此碘誘導的凋亡效應可能是碘參與甲狀腺癌發生、發展的一條重要途徑，但是其作用機理仍不清楚。

本文首先研究碘（I_2）誘導甲狀腺癌細胞的凋亡效應及其發生機理。研究發現：碘處理后的甲狀腺癌細胞 Bak 表達增加，膜電位明顯降低胞漿中細胞色素 C 增加，并且 caspase 3 激活，最終導致細胞凋亡。这种现象在高剂量碘（100 μM）的情况下尤为明显。此结果證明碘可以通過線粒體介導的凋亡途徑誘導甲狀腺癌細胞凋亡。研究同时發現：碘處理可以明顯降低甲狀腺癌細胞中突變型 p53 的表達，但是卻可以增加 p21 的表達。同時，高剂量碘可以激活 MAPKs 通路，包括 ERK1/2, p38 and JNK1/2。隨后的實驗中證實三者均具有抗凋亡作用。值得注意的是：抗凋亡分子 Bcl-xL 在高剂量碘誘導的凋亡過程中并不降低，相反，增加明顯。综合以上研究表明碘可以通過線粒體介導的細胞凋亡途徑誘導細胞凋亡，并且通過抗凋亡通路的 MAPKs 調節凋亡相關分子突變型 p53，p21 以及 Bcl-xL 的表達从而達到調節凋亡目的。這一結果為流行病學研究所得的結論---低劑量碘

攝取促進甲狀腺癌進展提供了分子學證據。同時也可能為甲狀腺癌的進一步治療提供了新的治療靶分子。

為了進一步探明碘在甲狀腺癌化療所誘導的凋亡中的作用，本研究以惡性程度最高的未分化型甲狀腺癌作為研究對象。研究中用甲狀腺癌化療常用藥物阿霉素（Dox）和新近使用的組蛋白脱乙酰基酶抑制劑類的丁酸鈉（NaB）單獨或者聯合誘導甲狀腺癌細胞凋亡，并觀察適當剂量的碘（I_2，50 μM）對這些化療藥物所誘導凋亡的影響。結果顯示單獨使用 Dox 或者 NaB 诱导的癌細胞死亡率均較低，而兩者聯合使用（NaB-Dox）可以大大提高藥物的的抗腫瘤效用。碘對于這兩種藥物的影響也截然不同：碘抑制 Dox 和 NaB-Dox 誘導的凋亡效應，卻明顯促進 NaB 誘導的凋亡效應。進一步的研究表明，碘進一步促進 NaB 降低 Bcl-xL 表達的能力，而削弱 Dox 降低 Bcl-xL 表達的能力。進一步的實驗證明這些不同的反應是通過 ERK 和/或 p38 調節的 Bcl-xL 的表達而实现。ERK 和 p38 之間的平衡決定了 Bcl-xL 的表達：ERK/p38 活性比例較高時 Bcl-xL 增加，腫瘤細胞則易于抵抗凋亡；而 ERK/p38 活性比例較低時 Bcl-xL 表達則降低，腫瘤細胞就易于凋亡。因此，碘通過調節 ERK/p38 介導的 Bcl-xL 表達从而達到調節化療藥物的作用。這一結果证实了碘在甲狀腺癌化療中具有重要意义。

總之，凋亡是碘參與甲狀腺癌發生和治療的一條關鍵途徑。其中 MAPKs 介導的 p53, p21 和 Bcl-xL 表達則是碘影響甲狀腺癌細胞增值和凋亡的重要分子。而在甲狀腺的化療中，碘又可以通過 ERK/p38 介導 Bcl-xL 表達而影響甲狀腺癌的治療效果。

PUBLICATIONS

Journal Article

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Review

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ABBREVIATIONS

%	Percentage
$\Delta\psi m$	Mitochondrial membrane potential
°C	Degree celsius
AIF	Apoptosis inducing factor
AIT	Apical iodide transporter
Apaf-1	Apoptotic peptidase activating factor 1
ATC	Anaplastic thyroid cancer
Bak	Bcl-2 homologous antagonist/killer
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BH	Bcl-2 homology domains
BRAF	V-raf murine sarcoma viral oncogene homolog B1
cAMP	Cyclic adenosine monophosphate
CTNNB1	β-catenin
Cyto C	Cytochrome C
DD	Death domain
DioC6	3,3′-dihexyloxacarbocyanine iodide
DISC	Death-inducing signaling complex
DIT	Di-iodotyrosine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin

DTC	Differentiated thyroid cancer
DTT	Dithiothreitol
DUOX	Dual oxidase
ECL	Enhanced chemiluminescence
EDTA	Ethlenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FADD	Fas-associated death domain
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FTC	Follicular thyroid cancer
G3PDH	Glyceradehyde-3-phosphate dehydrogenase
h	Hour
H_2O_2	Hydrogen peroxidase
HDAC	Histone deacetylases
HRP	Horse radish peroxidase
I^-	Iodide
I_2	Iodine, molecular iodine
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
MAPKs	Mitogen activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
mg	Milligram

min	Minute
MIT	Monoiodotyrosine
ml	Mililiter
mM	Milimolar per liter
MMP	Mitochondrial membrane potential
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide
NaB	Sodium butyrate
NF-κB	Nuclear factor kappa B
NIS	Sodium iodide symporter
nm	Nanometer
NP-40	Nonidet P-40
NTRK1	Neurotrophic tyrosine kinase receptor, type 1
NUE	NIS upstream enhancer
OMM	Out mitochondrial membrane
p-	Phospho-
p21	Cyclin-dependent kinase inhibitor 1A
p53	Tumor protein 53
Pax-8	Paired box-gene 8
PBS	Phosphate buffer saline
PDS	Pendrin syndrome
PI3K	Phosphoinositide 3-kinases
PMSF	Phenylmethy-sulphonyl-fluoride
PPARG	Peroxisome-proliferator-activated-receptor-γ

PTC	Papillary thyroid cancer
RAS	A family of genes encoding small GTPases
RET	Proto-oncogene encodes a receptor tyrosine kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SD	Strandard deviation
SDS	Sodium dodecyl sulfate
siRNA	Small interfering ribonucleic acid
STAT	Signal transducers and activator of transcription
T3	3,5,3' triiodothyronine
T4	3,5,3',5' tetraiodothyronine
TTF-1	Thyroid transcription factor 1
TG	Thyroglobulin
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TPO	Thyroid peroxidase
TRAIL	TNF-related apoptosis-inducing ligand
TSH	Thyroid-stimulating hormone; thyrotropin
TSHR	TSH receptor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UDTC	Undifferentiated thyroid cancer

µg	Microgram
µL	Microliter
µM	Micromolar per liter

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CHAPTER I: GENERAL INTRODUCTION

1.1 Thyroid cancer

Thyroid cancers arising from follicular cells have classically been classified into following categories: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and undifferentiated or anaplastic thyroid cancer (ATC). Of the three, PTC is the most common and accounts for more than 80% of thyroid cancers, while FTC for approximately 10% and ATC for about 1-2%. PTC and FTC are generally regarded as well-differentiated thyroid cancers (DTC) and ATC is undifferentiated thyroid cancer (UTC) (Sherman, 2003).

1.1.1 Incidence

Thyroid cancer accounts for 1% of all human cancers diagnosed worldwide, with about 2.59% of all new malignant tumors diagnosed annually (Jemal et al., 2008). It is rare compared to other cancers, but is the most common endocrine malignancy. Recently, the worldwide incidence of thyroid cancer has increased over a period of several decades in various countries (Kilfoy et al., 2009b), notably in the United States (Davies and Welch, 2006), Canada (Liu et al., 2001) and France (Colonna et al., 2007), although the advantage in the pathologic method for tumor detection may partly contribute to the higher incidence (Grodski et al., 2008).

1.1.2 Risk factors

Epidemiology studies on thyroid cancer incidence and histological analysis have been widely used to locate risk factors. Several associated risk factors in thyroid cancer generation or development have been identified, for instance, age, gender, radiation exposure, and iodine deficiency, which will be described in detail as follows.

1.1.2.1 Age

Thyroid cancer is the most heterogeneous disorder that affects all age groups from children through seniors, and almost half (48%) of all cases occur in people aged less than 50 years. The majority of cases occur between 25 and 65 years of age, while age of more than 45 years or less than 25 years is a particularly strong independent prognostic factor (Duntas and Grab-Duntas, 2006).

1.1.2.2 Gender

Gender is one of the few known risk factors of thyroid cancer. Thyroid cancer is more common in female than male, and the ratio between them is approximately close to 3:1 (Glattre and Kravdal, 1994). The female predominance suggests that hormonal factors may be involved. Some studies suggest that gender is an age-specific effect modifier for thyroid cancer (Kilfoy et al., 2009a) (Fig. 1.1), and the biological changes that occur during pregnancy may increase the risk of thyroid cancer (Brindel et al., 2008).

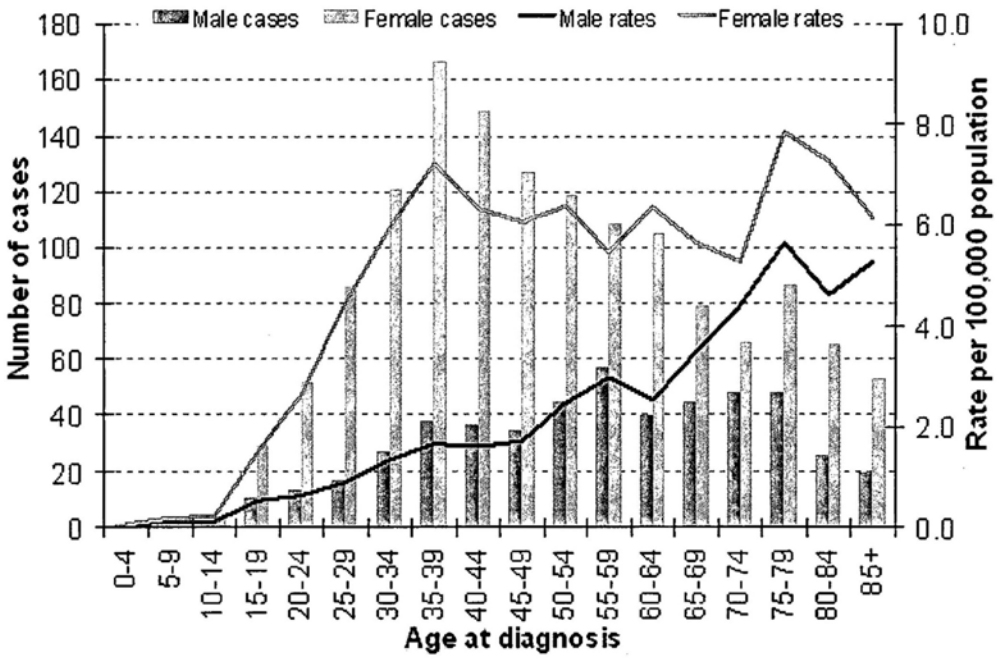


Fig. 1.1 The age- and gender-specific cases and rates for thyroid cancer. In the UK, thyroid cancer is rare in children, while in adults the incidence rates rise steadily with age. Although the rates are highest in the over 75s, there is a substantial number of cases at younger adult age. Female predominance appears in each age-specific group. (Http://info. cancerresearchuk.org/cancerstats/types/thyroid/incidence/, access date August 2009)

1.1.2.3 Radiation exposure

Thyroid cancer is hypothesized to result from mutational events combined with growth stimulation. It is likely that radiation is the main cause in activation of mutational events in thyroid cancer. Radiation exposure, including radiation therapy for malignancy, is the only factor that has been shown unequivocally to cause thyroid cancer in adults and children, especially when the exposure occurs in childhood (Dal Maso et al., 2009; Richardson, 2009).

1.1.2.4 Iodine deficiency

Chronic iodine deficiency is firmly established as a risk factor in thyroid cancer (Dal Maso et al., 2009; Delange and Lecomte, 2000; Feldt-Rasmussen, 2001; Lind et al., 2002). Moreover, iodine deficiency could influence tumor morphology and aggressiveness of thyroid cancer (Williams et al., 2008), leading to a changing pattern of less prevalent PTC but more aggressive ATC, although the point is still controversial (Dijkstra et al., 2007; Knobel and Medeiros-Neto, 2007).

1.1.3 Therapy

Patients with thyroid cancer all need active therapies because most of them have favorable prognosis if the correct therapy projects proceed on time. At present, three

main types of the thyroid cancer therapy are popular, surgery resection, radioactive iodide therapy, and chemotherapy. They will be presented in detail as follows.

1.1.3.1 Surgery

Surgery is usually the first treatment for thyroid cancers, particularly for DTC. When the cancer is removed early, most of patients have an excellent chance of being cured completely. However, the role of surgery in patients with ATC is controversial and depends on the extent of disease at presentation. Unfortunately, the vast majority of these patients have disease beyond the bounds of any meaningful resection. And the surgery alone does not have the potential to alter the course of the disease.

1.1.3.2 Radioactive iodide therapy

Thyroid cancer is particular among cancers because thyroid cancer cells are unique of the human body that could absorb iodide. Most thyroid cancer cells retain their abilities to absorb and concentrate iodide, which provides a perfect treatment method for radioactive ^{131}I therapy. ^{131}I therapy is one of the most effective treatments for thyroid cancer, particularly for those with distant metastases (Kuo et al., 2009; Sampson et al., 2007; Van Nostrand and Wartofsky, 2007). But ATC is relatively radioresistant and even no benefit with radiotherapy (Pasieka, 2003).

1.1.3.3 Chemotherapy

Surgery and radiotherapy tend to work better for thyroid cancer, but chemotherapy is sometimes used to treat advanced thyroid cancer especially for patients with non-radioiodide avid thyroid cancer. Chemotherapy uses antitumor drugs, such as doxorubicin and cisplatin, to destroy cancer cells by disrupting the growth of cancer cells. However, the effect of therapy is limited due to the high side effects and chemoresistance (Matuszczyk et al., 2008).

1.1.4 Prognosis

Thyroid cancers exhibit the full range of malignant behavior from the relatively indolent occult DTC to uniformly aggressive and lethal ATC. Among younger patients, both PTC and FTC have more than 97% cure rates if treated appropriately. But, the transformation of DTC to ATC is supported by pathological and molecular evidence (Hunt et al., 2003; Nikiforov, 2004). ATC grows rapidly to replace normal thyroid tissue. It spreads locally by invading adjacent structures, and metastasizes to regional lymph nodes and distant organs. Patients with ATC have a uniformly poor prognosis irrespective of the treatments given, resulting into the median survival time is 4 to 12 months (Are and Shaha, 2006). Thus, the strategy for preventing the progression of DTC into ATC and elevating chemotherapy effect on ATC should lead to improvement in the overall prognosis of thyroid cancer.

1.2 Iodine and thyroid cancer

Iodine is an essential element of body, which could be obtained from food, water and chemical agents or iodized salt, etc. In healthy adults, the total amount of iodine in body is about 30-50 mg: more than 70% of iodine is concentrated in thyroidal tissues, while the other less 30% of iodine is concentrated in extrathyroidal tissues. Due to approximately 90% of iodine is excreted in the urine, iodine status is usually assessed by measuring urinary iodine concentration. The accepted minimum adequate level of urinary iodine is 100 μg/L, and the levels above this are considered normal. Iodine in thyroid tissue works as the key constituent of thyroid hormone thyroxin 3,5,3',5' tetraiodothyronine (T4) and 3,5,3' triiodothyronine (T3), both of which play important roles in cell growth and development. Iodine accounts for 65% of the molecular weight of T4 and 59% of the T3. While, iodine in non-hormonal form is concentrated in extrathyroidal tissues, such as mammary glands, eyes, gastric mucosa, the cervix, and salivary glands.

1.2.1 Iodine in thyroid cancer formation

The mechanisms of carcinogenesis in thyroid tissues are complicated and unclear. Many etiological risk factors have been identified for thyroid cancer, such as radiation exposure, reduced iodine intake, female hormones and inherited conditions (Dal Maso et al., 2009; Gimm, 2001). The specific mechanisms of cancer formation are unclear, but

what all cancers do share is their ability to proliferate beyond the constraints that normally limit tissue growth. Aberrations in the regulation of a number of key pathways which control cell proliferation and survival are mandatory for the establishment of all cancers (Evan and Vousden, 2001).

Aberrant growth and mutation stimulation are major prerequisites for carcinogenesis (Krohn et al., 2007). Thyroid follicular cells proliferate slowly under normal conditions, which allow them to endure and accumulate a variety of insults. Radiation is an example of a gene mutation stimulator in thyroid cancer. The predilection of the thyroid gland to radiation-induced injury seems to be closely linked to chromosomal rearrangement and aberrant gene activation (Baverstock et al., 1992; Belfiore et al., 2001; Williams, 2002).

Iodine may theoretically act as aberrant growth stimulator in the formation of thyroid cancer. Animal experiments have shown that mice fed an iodine-restricted diet have an increased chance of developing thyroid cancer (Fortner et al., 1960; Schaller and Stevenson, 1966). In one long-term study, rats were given either a high, normal or low iodine diet for 110 weeks. The result showed that the proliferation rate of thyroid cells increased significantly by between 5- and 30- fold over normal under both the high and low iodine diets. Although the increased proliferation rate of thyrocytes induced by iodine only lead to the formation of adenomas, in the presence of radiation these iodine-mediated changes can progress into cancers (Boltze et al., 2002). In another study, the tumor-promoting effects of an iodine deficiency were enhanced when a chemical

carcinogen was present (Kanno et al., 1992). Therefore, both iodine deficiency and excess seems to promote cell division. With an increased proliferation rate, dividing cells may accumulate a higher number of genetic alterations (Belfiore et al., 2001; Boltze et al., 2002), making thyrocytes vulnerable to mutagens such as radiation and oxidative stress (Maier et al., 2007; Nishikawa et al., 2005; Williams et al., 2008).

High concentrations of hydrogen peroxidase (H_2O_2) and iodine are needed for the process of thyroid hormone synthesis (De Deken et al., 2002; Dupuy et al., 1991; Song et al., 2007). Besides being a substrate for hormone synthesis, H_2O_2 is a major source of free radicals or reactive oxygen species (ROS) which can cause substantial damage to a cell (Krohn et al., 2007; Song et al., 2007). Iodine is known to be able to function as an antioxidant (Smyth, 2003; Venturi et al., 2000), and when deficient, it affects the concentration of H_2O_2, as the generation of H_2O_2 has been shown to be inhibited by iodine *in vivo* and *in vitro* (Cardoso et al., 2001; Ohayon et al., 1994). Therefore, low levels of iodine can result in DNA damage and mutations by promoting ROS (Cooke et al., 2003; Kupper et al., 2008; Maier et al., 2007).

In summary, iodine deficiency is an important risk factor in the etiology of thyroid cancer. Iodine deficiency can cause the aberrant proliferation of thyrocytes and increase their sensitivity to ionizing radiation. At the same time, when functioning as an antioxidant, iodine deficiency increases the oxidative burden of the cell and thus increases the opportunity for DNA damage and mutations to occur.

1.2.2 Iodine and thyroid cancer transformation

Transformation is a term commonly used to describe the biological process in which normal or premalignant cells undergo a change to malignancy (Fox and Klawansky, 2006). In thyroid cancer, anaplastic transformation describes the intratumoral evolution or progression from DTC to ATC (Wiseman et al., 2007; Wiseman et al., 2003).

Histological and immunohistochemical studies indicate that ATC has close correlations with DTC, and that DTC may dedifferentiate to become more aggressive, or even transform into an anaplastic variety (Mooradian et al., 1983; Ozaki et al., 1999; Takeshita et al., 2008; Wang et al., 2007). Specifically, transformation is a terminal event, with ATC representing the end-point of thyroid cancer evolution (Wiseman et al., 2003). These findings have also been found at a molecular level, although the mutations responsible for anaplastic transformation are not completely understood (Hunt et al., 2003; Rodrigues et al., 2004).

Epidemiological data indicate that iodine is involved in the transformation of thyroid cancers. The worldwide incidence of thyroid cancer has steadily increased over the past few years (Kilfoy et al., 2009b), notably in the United States (Davies and Welch, 2006), Canada (Liu et al., 2001) and France (Colonna et al., 2007). An absolute increase in the overall incidence of thyroid cancer is still controversial because of changes in diagnostic vigilance, clinical practice and gender prevalence (Colonna et al., 2007; Davies and Welch, 2006; Grodski et al., 2008; Hodgson et al., 2004; Leenhardt et al., 2004; Liu et al.,

2001). Most investigators indicate that the incidence of various histological subtypes of thyroid cancer differ significantly, with an increase in PTC and a decrease in ATC (Dijkstra et al., 2007; Guan et al., 2009; Harach and Ceballos, 2008; Lind et al., 1998). The introduction of salt iodination has led to an improvement in the dietary supply of iodine and is considered to be generally beneficial. Thyroid cancers now have a more favorable prognosis, although a few studies dispute these findings (Pettersson et al., 1996; Sehestedt et al., 2006). Generally speaking, available data clearly show that the benefits of correcting a dietary iodine deficiency far outweigh the risks of iodine supplementation (Delange and Lecomte, 2000).

The mechanisms by which iodine is involved in the anaplastic transformation of thyroid cells remain unclear. Both thyroid-stimulating hormone (TSH) and radiation may play an important role in this process (Kapp et al., 1982; Mooradian et al., 1983; Wiseman et al., 2003). Iodine may act indirectly in anaplastic transformation by modulating TSH levels or by increasing thyroidal sensitivity to ionizing radiation.

1.2.3 Iodine and thyroid cancer apoptosis

It is well known that iodine is actively concentrated in the thyroid gland for the thyroid hormone biosynthesis, and that its regulation maintains normal thyroid homeostasis. However, iodine also plays an important role in thyroid cell proliferation and apoptosis (Smerdely et al., 1993; Tramontano et al., 1989; Vitale et al., 2000). There are many clinical conditions that are caused by either a deficiency or excess of iodine.

One of the most obvious clinical manifestations of an iodine deficiency is thyroid gland enlargement or goiter. The enlargement is caused partly by an increase in cell proliferation which can provide a favorable milieu for tumor development (Boltze et al., 2002; Eskin et al., 1975). An excess iodide intake is possible in certain circumstances, for example, in the therapeutic use of iodide-rich compounds such as amiodarone or iodinated radiographic contrast agents, leading to the release of a large amount of iodide and the induction of its toxic effects on the thyroid gland (Mahmoud et al., 1986; Martino et al., 2001; Unger et al., 1993).

In vitro experiments show that high concentrations of iodide inhibits FRTL-5 thyroid cell proliferation by arresting the cell cycle in the G0G1 and G2M phases (Smerdely et al., 1993). It is however uncertain whether iodide has a direct effect on FRTL-5 cell proliferation or whether its effects are mediated via other hormonal substances or growth factors. TSH, which is a notable regulator of thyroid cell proliferation and iodine metabolism, has been shown to exert an inhibitory effect on TSH-induced cAMP production (Filetti and Rapoport, 1983). It is also possible that the effect of iodine is mediated via transforming growth factor β (TGF-β), which can be induced by iodine in thyroid cells to inhibit the G1-S-phase transition (Pang et al., 1992; Yuasa et al., 1992). In addition to the role played by hormones and growth factors in the regulation of thyroid cell proliferation, autoregulatory effects of ambient iodine on thyroid cell functions are also possible (Bidey, 1990).

Besides cell cycle arrest, iodine also induces apoptotic phenomena in thyroid cells. Iodide-induced apoptosis shows a dose-dependent cytotoxicity in both immortalized thyroid cell-line (TAD-2) cells and primary thyroid cells, whereas it has no effect on non-thyroid cells (Vitale et al., 2000). Using rat thyroid FRTL-5 cells and primary dog thyrocytes, experiments demonstrate that FRTL-5 cells undergo apoptosis while iodide-treated primary dog thyrocytes do not, and thus the effect seems to be species specific (Golstein and Dumont, 1996).

The mechanisms involved in iodide-induced apoptosis are still unclear. During iodine treatment, ROS and lipid peroxide levels increased remarkably. At the same time, apoptosis induced by iodide in thyroid cells can be inhibited by propyl-thiouracil, an inhibitor of peroxidase, suggesting that the effects depend on the formation of free radicals, excess molecular iodine (I_2) generation, and the oxidation of ionic iodine by endogenous peroxidases (Smyth, 2003; Vitale et al., 2000). NIS mediates iodide transport while TPO mediates intracellular iodide retention in the normal thyroid cell and incorporates iodide into the protein (Dohan et al., 2003; Huang et al., 2001). Based on this evidence, iodide has been used to treat NIS/TPO gene-modified lung cancer cells. The results have demonstrated that the therapeutic dose of nonradioactive iodide can induce apoptosis in genetically modified lung cancer cells with an increase in ROS levels *in vitro*, and limit the growth of NIS/TPO-modified tumors *in vivo* (Zhang et al., 2003). Interestingly, molecular iodide can induce a time- and dose-dependent apoptosis without involvement of NIS and peroxidase activity (Garcia-Solis et al., 2005). Therefore, it

appears that iodide-induced apoptosis may be mediated by molecular iodide or iodinated derivatives, iodolactones, and not by iodide directly (Langer et al., 2003; Shrivastava et al., 2006). In addition, iodine has been shown to induce apoptosis by activating mitochondria, with a decrease in the expression of anti-apoptotic proteins and an increase in p21. However, this mitochondrial-mediated apoptosis is independent of caspases (Shrivastava et al., 2006; Upadhyay et al., 2002; Zhang et al., 2003). One report indicates that apoptosis induced by iodine is independent of p53, which does not correlate with the modulation of bax, bcl-2 and bcl-xl expression (Vitale et al., 2000). However, the known underlying signaling pathways are limited. The genetic alterations correlated with apoptosis or cell proliferation signaling pathways in thyroid cancer as follows: RAS mutation, BRAF mutation or rearrangement, RET rearrangement, TP53 mutation, peroxisome-proliferator-activated-receptor-γ (PPARG) rearrangement, neurotrophic tyrosine kinase receptor, type 1 (NTRK1), β-catenin (CTNNB1) mutation, and so on (Kondo et al., 2006). The underlying molecular mechanism responsible for the action of iodine appears to be complicated (Tab. 1.1). Iodine may interfere with multiple signaling pathways including mitogen activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB) signaling pathways. Nevertheless, the mechanisms of iodine-induced apoptosis have not been clearly elucidated. Specifically, the pathways and key proteins involved in the process remain unknown, as do the conditions under which iodine induces apoptosis in thyroid cancers instead of controlling normal thyroid homeostasis. Further

research into the mechanisms of iodine regulation is therefore essential for our understanding of thyroid cancers.

Tab. 1.1 Interrelations among iodine, genetic defects, transformation, cell proliferation and signaling pathways in thyroid cancer

Genetic alterations	Expression			Radiation	Iodine	Iodide transport-related genes	Transformation or differentiation	Signaling pathways	Prognosis
	PTC	FTC	ATC						
BRAF mutation or rearrangement	29-83%	0	10-20%	Yes	Yes	Yes	Yes	Yes, such as MAPKs, PI3K or NF-κB pathway	Poor
RET rearrangement	13-43%	No	Low	Yes	Unknown	No	Yes	Yes, such as MAPKs, NF-κB or STAT3 pathway	Poor
RAS mutation	0-21%	40-53%	6-51%	No	Yes	Yes	Yes	Yes, such as MAPKs or PI3K pathway	Poor
NTRK1	0-50%	Unknown	Unknown	Yes	Unknown	Unknown	Yes	Unknown	Poor
TP53 mutation	0-5%	0-9%	55%	No	Unknown	Yes	Yes	Yes, p53 pathway	Poor
PPARG rearrangement	0	25–63%	0	Unknown	Yes	Yes	Yes	Yes, such as NF-κB pathway	Favorable
CTNNB1 mutation	0	0	4-61%	Unknown	Unknown	Yes	Yes	Yes, such as PI3K,Wnt/beta-catenin pathway[58]	Poor

Continued:

Genetic alterations	References
BRAF mutation or rearrangement	(Xing, 2005); (Henderson et al., 2009); (Nikiforova et al., 2003); (Takano et al., 2007); (Takahashi et al., 2007); (Guan et al., 2009); (Knauf et al., 2005); (Romei et al., 2008); (Durante et al., 2007); (Leboeuf et al., 2008); (Henderson et al., 2007), (Liu et al., 2008); (Namba et al., 2007); (Mitsiades et al., 2007); (Mian et al., 2008); (Durand et al., 2009).
RET rearrangement	(Henderson et al., 2009); (Kondo et al., 2006); (Rabes et al., 2000); (Nakashima et al., 2007); (Hamatani et al., 2008); (Romei et al., 2008); (Gujral et al., 2008); (Henderson et al., 2007); (Namba et al., 2007); (Kim et al., 2008); (Kim et al., 2007a); (Durand et al., 2009).
RAS mutation	(Kondo et al., 2006); (Smallridge et al., 2009); (Suchy et al., 1998); (Shi et al., 1991); (Garcia-Silva and Aranda, 2004); (Vitagliano et al., 2006); (Melillo et al., 2005); (Cass and Meinkoth, 2000); (Liu et al., 2007); (Hou et al., 2007); (Garcia-Rostan et al., 2003).
NTRK1	(Frattini et al., 2004); (Brzezianska et al., 2006); (Brzezianska et al., 2007); (Beimfohr et al., 1999); (Musholt et al., 2006); (Musholt et al., 2000).
TP53 mutation	(Kondo et al., 2006); (Smallridge et al., 2009); (Suchy et al., 1998); (Barzon et al., 2005); (Moretti et al., 1997); (Ito et al., 2006); (Whibley et al., 2009); (Bachmann et al., 2007).
PPARG rearrangement	(Kondo et al., 2006); (Nikiforov, 2008); (Tepmongkol et al., 2008); (Espadinha et al., 2007); (Lui et al., 2005); (Kato et al., 2006); (Sahin et al., 2005).
CTNNB1 mutation	(Kondo et al., 2006); (Smallridge et al., 2009); (Kim et al., 2007c); (Guigon et al., 2008); (Kitamura et al., 2000); (Nikiforov, 2004); (Abbosh and Nephew, 2005); (Abbosh et al., 2007); (Garcia-Rostan et al., 2001).

CTNNB1, β-catenin; NTRK1, neurotrophic tyrosine kinase receptor, type 1; PPARG, peroxisome-proliferator-activated-receptor-γ

1.2.4 Iodide metabolism in thyroid cells

Being the critical component of T3 and T4, iodine represents an essential element in thyroid physiology. The process of iodide metabolism is complex and includes iodide transport, iodide organification, thyroid hormone synthesis and secretion, and thyroid intermediary metabolism. The first step of iodide thyroid metabolism is represented by the thyroid trapping and concentration of iodide from blood, which is achieved by an active, energy dependent transport process across the basolateral plasma membrane of the thyrocytes (Fig. 1.2) (Baker and Morris, 2004; Schlumberger et al., 2007). Being responsible for the accumulation of iodide into thyrocytes, the protein is the sodium-iodide symporter (NIS) which is located at the basolateral membrane (Dai et al., 1996; Smanik et al., 1997). The proteins ensuring apical iodide efflux to the follicular lumen are pendrin (Scott et al., 1999) and an apical iodide transporter (AIT) (Rodriguez et al., 2002). Then, iodide is organified in the tyrosyl residues of thyroglobulin (TG) in a reaction catalyzed by thyroid peroxidase (TPO), in the presence of H_2O_2 which is produced by dual oxidase (DUOX). This occurs in the apical membrane of the thyrocytes facing the colloid. Each tyrosin molecule can take up to four iodide atoms which form the different types of thyroid hormone and are stored in colloid. Under stimulation by thyrotropin (TSH), the gland receives the signal and consequently releases thyroid hormones. Following secretion into blood, the predominant thyroid hormones, T4 and T3, are deiodinated by specific enzymes located mainly in the liver and kidney; thus, free iodine is released. Serum iodine is then passively filtered through kidney where it is excreted.

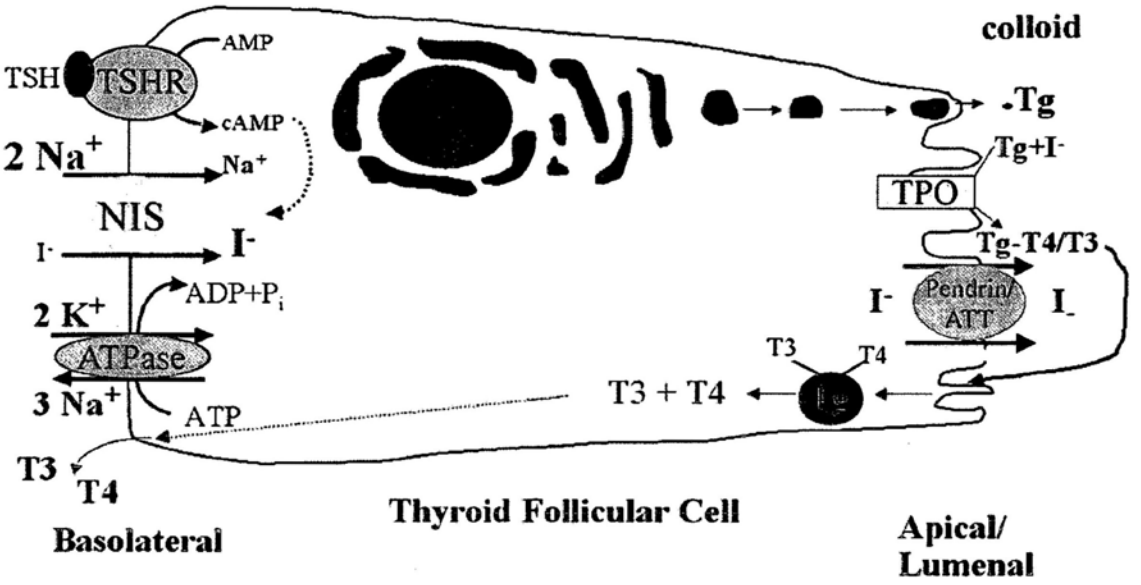


Fig. 1.2 Iodide metabolism in normal thyroid cells. TSH signaling via the TSH receptor (TSHR) controls thyroid hormone synthesis and can increase expression of NIS in the basolateral membrane of thyrocytes. As shown in the thyrocyte on the right, NIS uptakes iodide from the blood. The proteins involved in efflux of iodide at the apical membrane are pendrin and AIT. Iodide is organified in the tyrosyl residues of TG in a reaction catalyzed by TPO, in the presence of H_2O_2 which is produced by DUOX. TG contains MIT, DIT, T3, and T4 and is stored in colloid until T3 and T4 need to be released into the blood.

1.2.5 Defects of iodide metabolism in thyroid cancer

Iodide is an essential element for normal human function. Approximately 70% of the total body pool of iodine is stored in the thyroid gland. Iodide is actively transported into follicular thyroid cells by iodide transporters. Therefore, the ability of the thyroid cell to transport iodide, by iodide transporters, maintains the normal homeostasis of thyroid tissues. This ability is also a fundamental prerequisite for the effective use of radioactive iodine in the diagnosis and treatment of patients with thyroid cancers (Dadachova and Carrasco, 2004; Riesco-Eizaguirre and Santisteban, 2006).

The evidence that iodide transport is present in thyroid cancer tissues has been the basis for the use of radioiodine in the diagnosis and treatment of patients with thyroid cancer (Baker and Morris, 2004). Depending on the levels of dedifferentiation, thyroid cancer origins from follicular cells usually have defects in some biological properties of normal thyroid cells, such as TG production and iodide uptake, which result in decreased iodide bioavailability including radioactive isotopes of iodide (Mian et al., 2008). The two major steps of iodide metabolism—uptake and organification—are altered in thyroid cancer tissues (Schlumberger et al., 2007). Abnormality of NIS in thyroid cancer is the most popular defects in iodine metabolism, which could be caused by decreased NIS expression, NIS gene alterations and NIS location (Dohan et al., 2003; Kogai et al., 2000; Matsuda and Kosugi, 1997; Smanik et al., 1997). Besides abnormality of NIS, other factors such as TSH level in serum and TG expression can also regulate iodine uptake and organification aberrantly (Garcia-Jimenez and Santisteban, 2007; Lin, 2008). More importantly, the defective iodide-trapping mechanism appears to be an early and constant

feature of the oncogenic transformation of thyroid cells (Trapasso et al., 1999), and affects the prognosis of patients with thyroid cancer (Are and Shaha, 2006; Ward et al., 2003). Therefore, it is valuable to conduct researches to explore the mechanism of iodide transporters and their defects.

1.2.5.1 Sodium iodide transporter

NIS, an intrinsic membrane protein with 12 transmembrane regions, is a key plasma membrane protein expressed on the basolateral membrane of follicular cells. In thyroid, NIS mediates I^- uptake and it is the first step in the biosynthesis of the iodine-containing thyroid hormones (Baker and Morris, 2004; Riedel et al., 2001a).

A. Expression: Decreased NIS expression is one of the main factors responsible for the reduced iodine transport into thyroid cancer cells. Smanik *et al.* (Smanik et al., 1997) compared the expression of NIS in normal thyroid tissues to malignant thyroid tissues and showed that its expression was greatly reduced in thyroid tumors. Another report showed similar results, where the expression of NIS was decreased by 10- to 1200-fold in 40 of 43 thyroid cancers (Lazar et al., 1999). Further, investigations have revealed that a decrease in NIS expression occurs not only in primary tumors but also in metastases tissues (Arturi et al., 1998; Park et al., 2000). Mishra *et al.* (Mishra et al., 2007) studied the expression of NIS in thyroid cancer specimens from iodine deficient regions and found that the expression of NIS was detected in 73.3% of PTC and 70.0% of FTC but in only 33.3% of ATC. Therefore, these investigations have shown that the expression of NIS is decreased in thyroid cancer cells, and that its decrease is related to the subtype of thyroid cancers and to the dietary availability of iodine. Although the expression of NIS

is relatively high in PTC and FTC (Mishra et al., 2007; Saito et al., 1998; Wapnir et al., 2003), the ability of these tumors to concentrate radioactive iodide is still impaired, suggesting that there are other mechanisms that influence the activity of NIS, such as genetic mutations and an impaired targeting of NIS to the plasma membrane of thyroid cells (Dohan et al., 2003; Kogai et al., 2000; Peyrottes et al., 2009).

Currently, ten point mutations (V59E, G93R, Q267E, C272X, T354P, G395R, R124H, frameshift 515X, Y531X, and G543E) and two deletion mutations (DelM143-Q323 and DelA439-P443) have been described in the NIS gene. They are nonsense, alternative splicing, frame-shift, deletion, or missense mutations of the NIS gene (De La Vieja et al., 2004; Fujiwara et al., 1997; Fujiwara et al., 1998; Kosugi et al., 1999; Kosugi et al., 2002; Kosugi et al., 1998; Matsuda and Kosugi, 1997; Pohlenz et al., 1997; Szinnai et al., 2006). Russo *et al.* (Russo et al., 2001) have studied point mutations and other genetic alterations that may be responsible for the altered NIS protein function in tumors that still expressed NIS transcripts. Tumoral cDNAs derived from five PTC and two FTC were analyzed by direct sequencing after polymerase chain reaction (PCR) amplification of the structural NIS gene. No mutations or other genetic abnormalities were found in any of the tumor samples examined. These data indicate that mutations or other genetic alterations of the NIS structural gene may not be a major cause of the reduced iodide uptake in DTC. This anomaly may be caused by the origins of these mutations, as some occur in patients with thyroid cancer and others in patients with congenital iodide transport defects. This argument may also challenge the direction of further research. The cell polarity and location of the protein are important in NIS activity. The location of NIS protein in thyroid cancers differs from that in normal thyroid tissues.

In normal thyroid tissues, NIS protein is expressed on the basolateral membrane, while in thyroid cancer it is expressed predominantly in the cytosol, resulting in defective targeting of NIS to the plasma membrane (Dohan et al., 2001; Kogai et al., 2000; Saito et al., 1998; Sodre et al., 2008; Wapnir et al., 2003). Thus, the loss of cell polarity may result in the impairment of functional NIS expression and iodide concentration. In conclusion, the reduced expression of NIS, impaired tissue polarity, and mutations will all influence iodine transport in thyroid cancer cells. Even though the reduced iodine transportation cannot be fully explained by these defects, work on the stimulation and restoration of NIS expression is of valid therapeutic value in thyroid cancer treatment.

B. Regulation: NIS expression can be up-regulated or down-regulated by different agents. Many agents have been used to enhance NIS expression and thus result in an increased iodine concentration in normal and thyroid cancer cells (Kogai et al., 2006). These agents are classified into several groups, including thyrotropin agonists (thyrotropin, human choriogonadotropin) (Arturi et al., 2002; Kogai et al., 2000; van Hoek et al., 2008), retinoic acid receptor agonists (retinoic acid) (Coelho et al., 2005; Kurebayashi et al., 2000), cytokines (TGF-β1, IL-1α, IL-1β) (Kawaguchi et al., 1997; Schumm-Draeger, 2001; Spitzweg et al., 1999) and histone decacetylase inhibitors (valproic acid, trichostatin A, FR901228) (Akagi et al., 2008; Catalano et al., 2005; Fortunati et al., 2004; Kitazono et al., 2001). On the other hand, iodine and TG are used to down-regulate NIS expression. The roles of thyrotropin, iodine and TG will be further discussed below as they are intimately related to thyroid function in humans.

a. Thyrotropin: Thyrotropin (TSH) is the primary regulator of thyroid function and has long been known to stimulate the accumulation of I^- by thyroid cells (Vassart and Dumont, 1992). Levy *et al* demonstrated that NIS protein expression was up-regulated by TSH *in vivo* (Levy et al., 1997). TSH can also increase NIS mRNA and protein expression in FRTL-5 thyroid cells *in vitro* (Kogai et al., 1997). Experiments have shown that TSH up-regulates NIS expression and I^- uptake activity via a cAMP pathway, primarily by stimulating NIS at transcriptional and post-transcriptional levels (Ohno et al., 1999; Riedel et al., 2001b; Weiss et al., 1984).

Studies demonstrate that TSH stimulates the NIS promoter and NIS upstream enhancer (NUE) at the transcriptional level. NUE stimulates transcription in a thyroid-specific and cAMP-dependent manner (Chun et al., 2004). The transcriptional factors paired box-gene 8 (Pax-8) and thyroid transcription factor 1 (TTF-1) are involved in NIS gene transcription (Endo et al., 1997; Ohno et al., 1999). Pax-8 is required for transcriptional activation and cAMP stimulation of the NUE (Taki et al., 2002). The induction of Pax-8 expression may promote a redifferentiation of thyroid cancer cells, including a partial recovery of iodide uptake, which would be a fundamental prerequisite for a radioiodine-based therapeutic approach to thyroid cancers (Presta et al., 2005). TTF-1 overexpression does not increase the expression of NIS mRNA and iodine uptake, although prolonged iodide retention and organification in tumors are observed (Furuya et al., 2004). In addition, Riedel *et al* found that TSH could regulate NIS expression at the post- transcriptional level (Riedel et al., 2001b). TSH, which is required for NIS to target or to be retained in the plasma membrane, increases the half-life of the NIS protein and modulates the phosphorylation pattern of NIS protein (Kogai et al., 2000; Riedel et al.,

2001b). Besides its effect on NIS expression, TSH also contributes to the regulation of thyrocyte differentiation by modulating thyroid gene levels (Bruno et al., 2005). Remarkably, the phosphatidylinositol 3-kinase (PI3K) signaling pathway is activated in the process of TSH-stimulated NIS expression, and the inhibition of the PI3K pathway increases the functional expression of NIS in thyroid cancers (Kogai et al., 2008). PI3K is an important regulator of many cellular events, including proliferation, apoptosis and transformation (Chang et al., 2003; Rivas and Santisteban, 2003). However, the PI3K pathway is always dysregulated in thyroid cancer (Paes and Ringel, 2008). Thus, while a complicated crosstalk may exist between iodine, NIS, TSH and signaling pathways, influencing the proliferation, apoptosis and transformation of thyroid cells, the exact mechanisms remain unclear (Tab. 1.1).

The expression of TSH receptor (TSHR), which responds to TSH stimulation, is decreased or even absent in thyroid cancers (Hoffmann et al., 2006; Matsumoto et al., 2008; Milas et al., 2007). Decreased TSHR expression may result in a reduced radio-iodine uptake and lead to a negative response to radio-iodine therapy. Importantly, a decreased TSHR expression may represent a degree of dedifferentiation of the thyroid cancer and correlate with a high proliferation rate (Brabant et al., 1991; Xing et al., 2003). Therefore, in studying TSH-modulated NIS expression, the expression of TSHR should be taken into account.

b. Thyroglobulin: Thyroglobulin (TG), a 660 kDa dimeric protein, is the glycoprotein precursor of thyroid hormones T3 and T4. It is produced by thyrocytes and then secreted into the follicular lumen where it is stored. In thyroid cancer, the expression

of TG mRNA is decreased in PTC and completely lost in ATC (Brabant et al., 1991; Elisei et al., 1994; Lin, 2008). Clinically, the measurement of serum TG is widely accepted to be the best available tool with which to follow the disease status of thyroid cancer patients (Giovanella, 2008; Spencer and Lopresti, 2008).

As previously discussed, TSH increases the expression of NIS. Contrary to this, TG suppresses the gene expression and activity of NIS (Kohn et al., 2001). *In vivo* studies have demonstrated that the suppression is due to a feedback effect of TG (Suzuki et al., 1998). To clarify this point, purified, salt-extracted bovine and human 19S follicular TG was added to a medium with FRTL-5 cells. Results showed that the levels of TTF-1, TTF-2 and Pax-8 were suppressed first, followed by the suppression of TG and NIS expression, supporting that TG and NIS are regulated by one or more of these transcription factors (Espadinha et al., 2007; Suzuki et al., 1999).

c. Iodine: Iodine itself is a critical factor in regulating the accumulation of I^- by the thyroid gland. The most famous example of this is the Wolff-Chaikoff effect which describes hypothyroidism caused by the ingestion of a large amount of iodine. The cause is due to a transient blockade of the organification of iodine secondary to the down-regulation of NIS resulting in decreased transportation of iodide into the thyroid cells (Eng et al., 1999; Leoni et al., 2008). Further experiments show that moderate doses of iodine can inhibit the expression of NIS mRNA *in vivo* (Uyttersprot et al., 1997). An *in vitro* study has also indicated that excess iodine does not decrease NIS mRNA in FRTL-5 cells but does decrease the expression of NIS protein, which suggests that in an *in vitro* thyroid cell model iodine modulates NIS, at least partly, at a post-transcriptional

level (Eng et al., 2001). Nonradioactive iodine down-regulates the expression of NIS mRNA and protein. Iodide-rich compounds such as amiodarone can also decrease NIS mRNA expression by nearly half at therapeutic concentrations (Yamazaki et al., 2007). In addition, recent studies using an *in vitro* model of internal radiation of normal thyroid cells showed that ^{131}I indeed reduced thyroid iodide transportation in a dose-dependent manner (Lundh et al., 2007; Meller et al., 2008; Postgard et al., 2002).

Iodide affects the expression of NIS in the fetal thyroid gland. In one experiment, female rats were given either an iodine deficient, a low iodine or an iodine supplemented diet for 3 months (Schroder-van der Elst et al., 2001). Under the supplemented condition, NIS mRNA increased 2- and 4-fold in the thyroid of fetuses with a low and iodine supplemented diet respectively. In the low iodine diet, NIS expression was up-regulated. Finally, subsequent iodine supplementation in previously iodine deficient fetuses did not lead to the restoration of a normal absolute iodide uptake. These results suggest that all adaptive or defense mechanisms against iodine deficiency are already present in the fetus (Schroder-van der Elst et al., 2001). *In vitro* experiments also showed similar results, suggesting that NIS regulation by iodine may occur through modulation of human choriogonadotropin (Li et al., 2007).

1.2.5.2. Pendrin and apical iodide transporter

Pendrin (SLC26A4), a highly hydrophobic transmembrane 780-amino acid protein, is encoded by the SLC26A4 gene which is mutant in Pendred syndrome (PDS) (Everett et al., 1997; Kopp et al., 2008; Scott et al., 1999). Pendrin that is highly expressed in thyroid tissues is localized to the apical membrane of thyroid follicular cells, and acts as an

iodide-specific apical transporter responsible for iodide efflux from thyroid cells (Bidart et al., 2000; Royaux et al., 2000; Yoshida et al., 2002).

The apical iodide transporter (AIT, SLC5A8) is a protein responsible for iodide transport from the thyrocyte through the apical membrane into the colloid lumen. The AIT gene is located on chromosome 12q23 and encodes a 610 amino acid protein sharing 46% of its identity (70% similarity) with human NIS (Rodriguez et al., 2002). The AIT has other functions in addition to its role in iodide transportation. It transports monocarboxylates and short-chain fatty acids by a sodium-couple mechanism (Gopal et al., 2004; Gopal et al., 2007), acts as a tumor suppressor (Ganapathy et al., 2005; Li et al., 2003), and triggers tumor cell apoptosis (Thangaraju et al., 2006).

A. Expression: In normal thyroid tissues, pendrin is localized at the apical pole of thyrocytes in contrast to the basolateral location of NIS. Immunostaining reveals a higher pendrin protein expression in thyroid tissues from patients with Grave's disease than in normal thyroid tissues. This suggests a correlation between pendrin abundance and increased iodide organification (Mian et al., 2001; Royaux et al., 2000). To investigate whether the pendrin gene is altered during thyroid tumorigenesis, pendrin gene and protein expression were studied using RT-PCR and immunohistochemistry respectively. Results showed that pendrin mRNA expression in thyroid cancer tissues was 2- to 1000-fold lower (median, 100-fold) than in normal thyroid tissues, and that pendrin immunohistochemical staining was weak or positive in only a minority of tumor cells (Bidart et al., 2000). Using RT-PCR, Arturi *et al.* (Arturi et al., 2001) analyzed the patterns of pendrin in thyroid cancer cell lines and a series of thyroid tumor tissues. They

showed that pendrin expression did not correlate with NIS expression. The data also indicated that pendrin was present in most of the DTC cell lines, but was reduced or absent in DTC tissues. Kondo *et al.* assessed pendrin expression by immunohistochemistry and found it to be negative in all follicular and papillary carcinomas (Kondo et al., 2003). However, another study found that pendrin protein was detectable in 73.3% and 76.7% of FTC and PTC respectively (Skubis-Zegadlo et al., 2005). The difference might be due to the antibodies used in the experiments as the high-affinity antigen-purified antibodies against the amino- and carboxyl-termini of the pendrin molecule are both located intracellularly. Results suggest that the preserved pendrin expression in DTC may have a different functional status as its targeting to the apical cell membrane is affected (Gillam et al., 2004; Porra et al., 2002; Skubis-Zegadlo et al., 2005).

Information on AIT expression in thyroid tissues is limited. Using immunohistochemistry, AIT staining was detected in only about 10% of follicles from normal thyroid tissues. In thyroid carcinomas, the mean and median AIT mRNA levels were significantly decreased. Expression of AIT protein was undetectable in most PTC, weak but detectable in most FTC and negative in ATC (Lacroix et al., 2004). Porra *et al.* demonstrated that AIT expression was selectively down-regulated by 40-fold in PTC, and that most of them were methylated (Porra et al., 2005).

***B. Regulation*:** Human thyrocytes cultured with or without TSH exhibit different expressions of pendrin at the transcriptional level. TSH treatment partly prevents a decrease in pendrin and, the expression of pendrin at transcriptional levels is 3-fold

higher in TSH-treated cells than in untreated cells (Porra et al., 2005). In addition, Suzuki *et al* and Royaux *et al.* demonstrated that the expression of pendrin mRNA could be significantly induced by low concentrations of TG, which differs from the regulation of NIS by TG (Kohn et al., 2001; Royaux et al., 2000; Suzuki and Kohn, 2006).

Few studies have been carried out on the regulation of AIT expression. Porra *et al.* reported that AIT expression, unlike that of NIS and pendrin, was not regulated by TSH at the transcriptional level and that it was independent of the state of activation or functional activity of the thyroid tissue (Porra et al., 2005).

Taken together, iodine is a key element in the regulation of human thyroid development and function. It is clear that iodine not only maintains normal thyroid cell homeostasis, but also has an influence on the development of thyroid cancer. More importantly, iodine can induce cell cycle arrest and apoptosis in thyroid cancer cells. Although the responsible mechanism is not clear and some controversies exist, current findings indicate that the stress in thyroid tissues, remaining normal or becoming malignant, is, at least partly, controlled by iodine. At the same time, a crosstalk of tumorigenesis, progression and apoptosis can be influenced by iodine. Iodine is not only a unique element that acts on tissues to maintain their normal homeostasis, but also has the ability to influence tumor generation, progression and apoptosis. Further studies on the role and regulation of iodine are essential for our understanding of the formation and progression of thyroid cancers and hence for our ability to prevent, diagnose and treat them.

1.3 Apoptosis and cancer

Apoptosis is an intrinsic cell death program that may occur in multicellular organisms to keep proper tissue homeostasis and development. Due to the balance between proliferation and cell death is a key factor in tissue homeostasis, an aberrant increase or decrease in apoptosis is observed in a variety of pathological conditions including cancer (Nicholson and Thornberry, 2003). Aborting apoptosis is regarded as one hallmark of cancer cells in which two fundamental lesions are to be the underlying pathogenesis of cancer. One is gene mutations that give rise to excessive proliferation; the other is a disruption of apoptotic signaling allows mutated cells to continue to proliferate and live beyond their normal life span perpetuating cycles of mutations and oncogenesis (Hanahan and Weinberg, 2000). Besides the role in carcinogenesis, apoptosis is feasible to cancer therapy because some cancer cells are sensitive to DNA-damaging agents or irradiation (Brown and Attardi, 2005; Johnstone et al., 2002). Moreover, resistance of apoptosis is also the critical cause that results into tumor therapy failure (Igney and Krammer, 2002). Therefore, apoptosis has emerged as an important process from carcinogenesis to cancer therapy.

1.3.1 Mitochondrial- and receptor-mediated apoptosis pathway

The apoptotic signaling events can be roughly divided into two major pathways—the mitochondrial-mediated pathway (the intrinsic pathway) and the receptor-mediated pathway (the extrinsic pathway) (Fig. 1.3). The mitochondrial-mediated apoptotic pathway occurs in response to a wide range of death stimuli including

activation DNA damage, chemotherapeutic agents and radiotherapy (Shi, 2001). Following damaging cellular stress, mitochondrial membrane collapse and mitochondrial membrane potential (MMP) decreased, then proteins of intermembrane space, such as cytochrome C (Cyto C), can then diffuse into the cytosol and lead to the activation of caspase enzymes that are responsible for apoptosis such as caspase 9 (Green, 2006; Green and Chipuk, 2008). The active Cyto C/Apaf-1/caspase 9 complex has been nicknamed the "apoptosome" and activates executioner caspases. Receptor-mediated apoptosis is activated by pro-apoptotic receptors tumor necrosis factor (TNF) superfamily of receptors on the cell surface by their associated ligands. Once being formed, the receptor-ligand complexes oligomerize will firstly bind the intracellular adaptor proteins, such as TNFα receptor associated death domain and Fas-associated death domain, and then recruit the inactive performs of certain members of the caspase protease family. The caspases are recruited to this death-inducing signaling complex---caspase 8 and caspase 10 which function as 'initiator'. Once being activated through mitochondria or receptor mediated pathway, the initiator caspases cleave and activate 'executioner' caspases, mainly caspase 3, 6, 7, triggering apoptosis (Igney and Krammer, 2002).

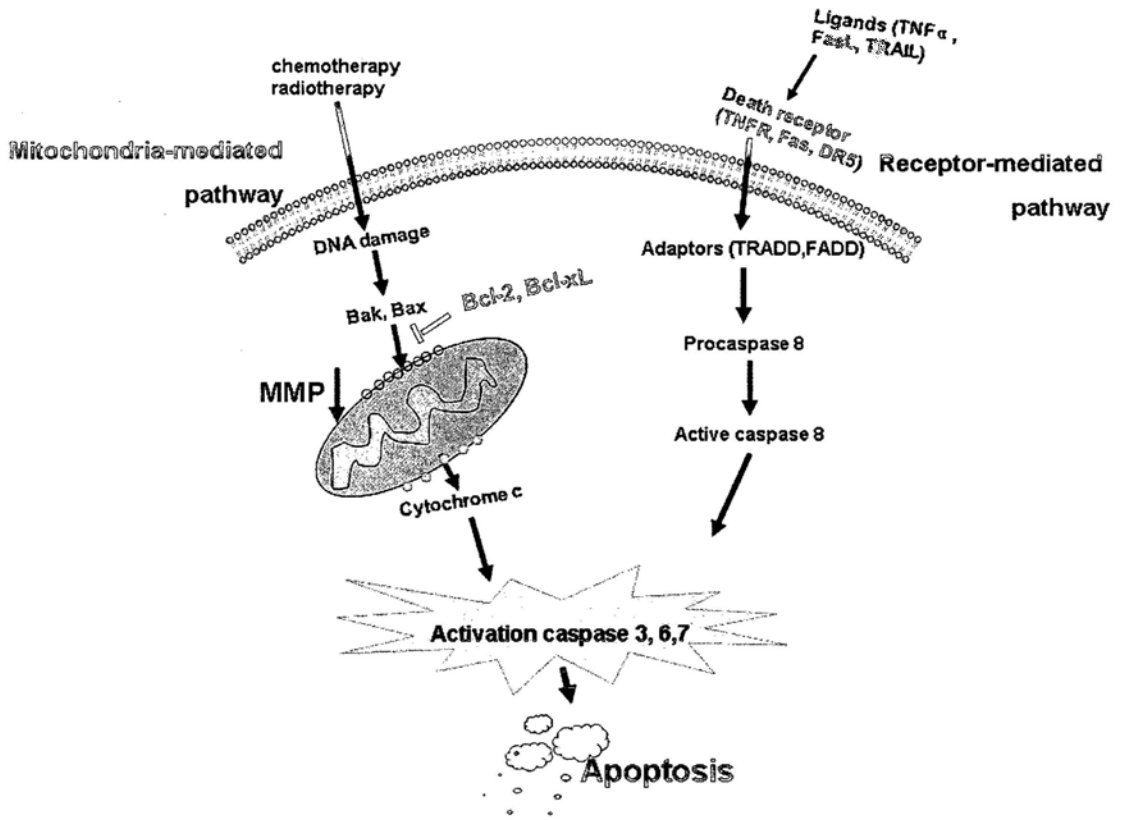


Fig 1.3 The two major apoptotic pathways: mitochondrial- and receptor-mediated apoptotic pathways. Apoptosis can be activated by two major pathways: mitochondrial- and receptor-mediated apoptotic pathways. The mitochondrial-mediated apoptosis pathway is initiated by various signals, principally extracellular stimuli, while the receptor-mediated apoptosis pathway is activated by Fas ligand or TNF-related apoptosis-inducing ligand (TRAIL). Both mitochondrial- and receptor-mediated apoptotic pathways converge on the so-called executioner canspases and trigger apoptosis. (Adapted from http://www.research apoptosis.com/apoptosis/pathways/index.m, access date August 2009)

In eukaryotic cells, mitochondria are the place not only supply cellular energy but also engage in cell proliferation and death (McBride et al., 2006). Because mitochondria play the key role in cell metabolism and the character of tumor cells resistant to apoptosis, researchers should consider such resistance related to the particular properties of mitochondria in cancer cells which are distinct from those in normal cells in many ways. For instance, alterations of energy-supplying pathways may suppress mitochondrial activity or impair mitochondrial function and disrupt membrane stabilization (Gogvadze et al., 2008). Meanwhile, iodine is the main constitution of thyroid hormone that could influence cell metabolism. Thus, mitochondria may be the critical point for iodine and thyroid cancer to establish communications through apoptotic pathway. Therefore, this study will focus on mitochondrial-mediated apoptotic pathway and its regulators in thyroid cancer formation and treatment.

1.3.2 Bcl-2 family proteins in apoptosis

Bcl-2 family proteins play crucial roles in regulating mitochondrial events during initiation of the apoptotic process. The Bcl-2 family proteins consist of both anti-apoptotic and pro-apoptotic proteins which possess at least one of four conserved Bcl-2 homology domains (BH1 to BH4) (Fig. 1.4) (Danial, 2007). The anti-apoptotic proteins contain the Bcl-2, Bcl-xL, Mcl-1, Bcl-w and etc, all of which have four Bcl-2 homology domains. The pro-apoptotic proteins, such as Bax, Bak and Bok, contain Bcl-2 homology 1-3 domains, whereas other pro-apoptotic proteins, such as Bid, Bad and Bim, contain only the BH3 domain (Fig. 1.4).

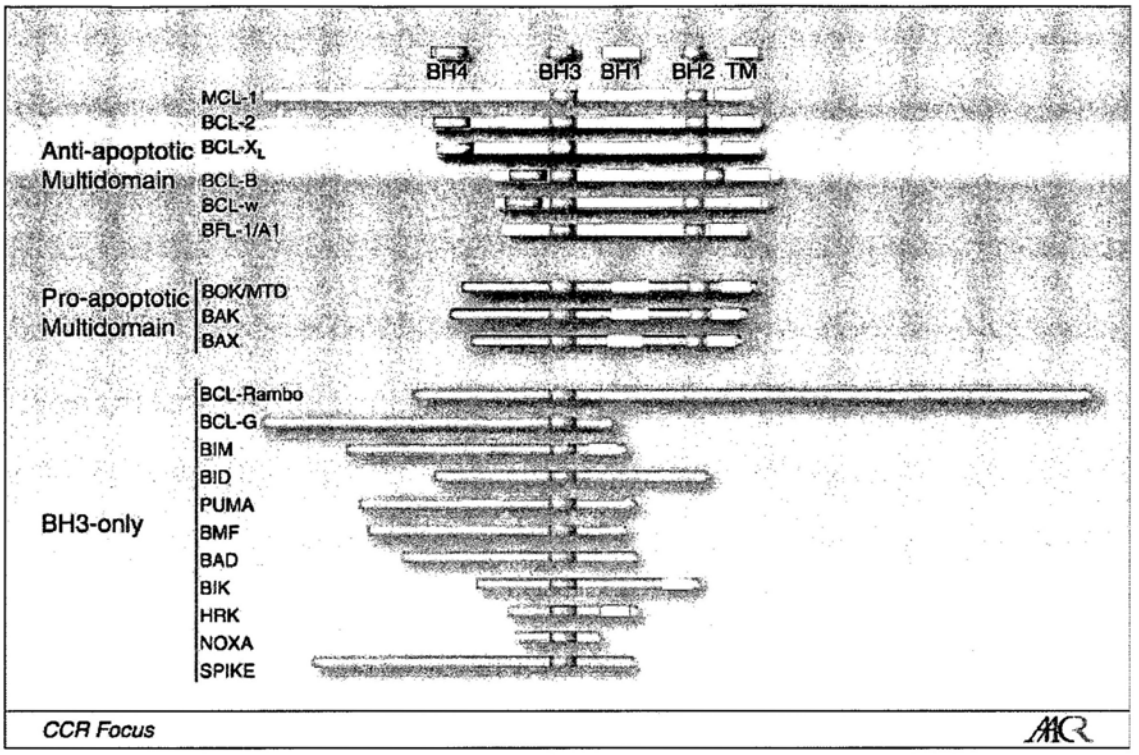


Fig. 1.4 Classification of Bcl-2 family according to conserved domains. Proteins of Bcl-2 family control the integrity of the out mitochondrial membrane (OMM). The pro-apoptotic effecter proteins Bak and Bax are responsible for permeabilizing the membrane, while the anti-apoptotic proteins Bcl-2 and Bcl-xL inhibit permeabilization of the membrane and BH3-only proteins promote it. When Bak and Bax are activated by BH3-only proteins in response to damaging cellular stress signals, they form complexes of either Bax-only or Bak-only molecules, and then assemble in the OMM causing MMP decreased. Thus, proteins localized between the outer and inner membranes, such as Cyto C and apoptosis inducing factor (AIF), and then initiate apoptosis (Chipuk and Green, 2008; Danial, 2007; Green, 2005; Green, 2006; Green and Chipuk, 2008).

1.3.3 MAPK cascades in apoptosis

A protein kinase is a kinase enzyme that modifies other proteins by adding phosphate to one of three amino acids that have a free hydroxyl group on them, resulting in a functional change of the target proteins (substrates) by changing enzyme activity, cellular location or association with other proteins (http://en.wikipedia.org/wiki/Protein_ kinase, access date August 2009). Proteins kinases, especially those involved in signal transduction, such as MAPKs pathways (Wada and Penninger, 2004), are well known cellular pathways involved in modulating apoptosis.

In mammals, MAPKs signaling pathways are well characterized pathways in regulating cell proliferation and survival. Both stress- and growth-regulating signals are transduced from the cell surface into the nucleus via these cascades. There are three well characterized subfamilies of MAPKs, including extracellular signal-regulated kinase (ERK1/2), p38 MAPK (p38α/β/γ/δ) and c-Jun amino-terminal kinase (JNK1/2/3) (Chang and Karin, 2001). MAPKs are activated by phosphorylation cascades. MAPK activity is activated and regulated via three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK) (Chang and Karin, 2001). MAPKK can be activated by more than one MAPKKK which is responsive to distinct stimuli. Each MAPKK is also responsive specifically for MAPKs activation through phosphorylation: MEK1/2 for ERK1/2, MKK3/6 for the p38 and MKK4/7 (JNKK1/2) for the JNK (Fig. 1.5) (Chang and Karin, 2001; Roberts and Der, 2007). During the process of MAPKs activation, the MAPKKK and MAPKK provide the chance for signal amplification and detecting input from other signaling pathways to

enhance or suppress the signal to the MAPKs. In addition, positive and negative autocrine loops triggered by the activating signal upstream of the MAPKs can also provide specific spatial patterns of MAPKs activation (Pearson et al., 2001). Once activated, MAPKs will influence cell proliferation and apoptosis mainly through modulating the expression of target proteins which include transcription factor p53, apoptosis regulators Bcl-2 family proteins, and etc (Balmanno and Cook, 2009; Cordenonsi et al., 2007; Grethe et al., 2006; Lei et al., 2002; Wada and Penninger, 2004; Yamamoto et al., 1999). All of these make MAPKs activation more important in regulation of cell survival and apoptosis. Thus, the pro- or anti-apoptotic functions of ERK, p38 or JNK depend on cell type, nature of the death stimulus, duration of their activation and the activity of other signaling pathways (Balmanno and Cook, 2009; Dhanasekaran and Reddy, 2008; Wada and Penninger, 2004).

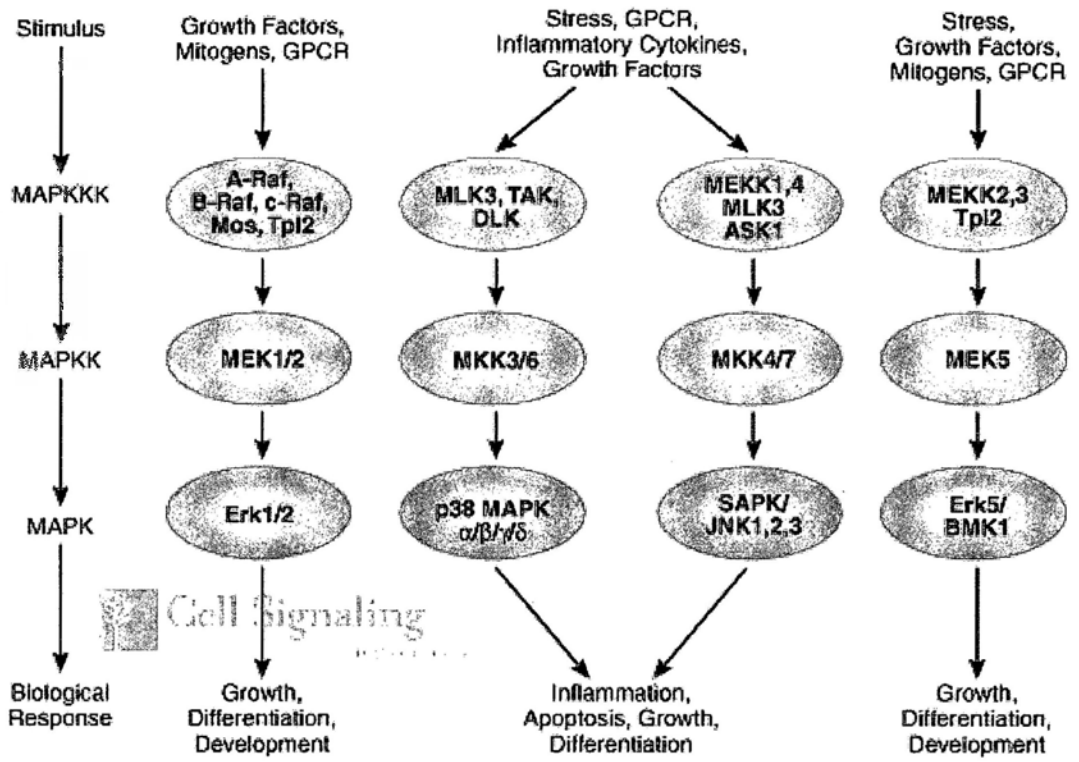


Fig. 1.5 Mammalian MAPK cascades. There are four major mammalian MAPKKK-MAPKK-MAPK protein kinase cascades. The ERK pathway is commonly activated by growth factors, whereas the JNK, p38 and ERK5 pathways are activated by environmental stress including osmotic shock, ionizing radiation. Many of the substrates for MAPKs are nuclear transcription factors and apoptosis regulators. Protein phosphatases remove the phosphates that were transferred to the protein substrate by the MAPKs. In this manner, the action of MAPKs and protein phosphatases reciprocally and rapidly alter the behavior of cells as they respond to changes in their environment (Johnson and Lapadat, 2002; Roberts and Der, 2007). (http://www.cellsignal.com/reference/pathway/MAPK_Cascades.html, access date August 2009)

1.3.4 Apoptosis in thyroid cancer development

Defects in apoptosis play important roles in tumorigenesis by way of a process of allowing tumor cells to survive beyond their normally intended lifespans, providing protection from stress as tumor mass expands, and admitting enough time for accumulative genetic alterations. The process deregulates cell proliferation, interferes with differentiation, and increases cell motility and invasiveness during tumor progression (Reed, 2003). Based on the past decades of studies on apoptosis and cancer development, it has been evidenced that besides the expression of proteins that promote cell proliferation (Evan and Vousden, 2001), tumor progression requires the expression of anti-apoptotic proteins of the inactivation of essential pro-apoptotic proteins (Igney and Krammer, 2002). In thyroid cancer, both genetic alterations activated aberrant cell signaling pathway in cell proliferation and impairment of apoptosis regulators play critical roles in tumorigenesis.

1.3.4.1. Genetic alterations in cell signaling pathway

Tumorigenesis and transformation are the consequence of a multistep process involving the accumulation of mutational epigenetic changes that promote cell proliferation and suppression of apoptosis. It has been proposed that thyroid cancers accumulate a number of alterations at the genomic level and the genomic instability has vital actions in the progression of thyroid cancer. The genetic alterations in thyroid cancer are as follows: RAS mutation, BRAF mutation or rearrangement, RET rearrangement, TP53 mutation, PPARG rearrangement, NTRK1, CTNNB1 mutation, and so on (Kondo et al., 2006). Meanwhile, the distribution of these molecular features in DTC and ATC

are different. For instance, several mutations occurring in PTC (e.g., RAS and BRAF) are also seen in ATC which suggests these are early events. Other mutations like p53 and CTNNB1, they just contribute to the extremely aggressive behavior of ATC, which suggest that these mutations are late events (Nikiforov, 2004; Smallridge et al., 2009; Sobrinho-Simoes et al., 2005). Thus, signaling through growth factors and their receptors is considered essential for thyroid cancer progression, and some of these growth factors have been identified as modifiers of the behavior of transformed thyroid cells.

Both of RAS and RAF are both recruited to stimulated growth factor receptors. They participate in signal transduction through RAS-RAF-MEK-ERK-MAP kinase pathway to modulate cell proliferation, differentiation and survival by regulating apoptosis regulators such as Bcl-2 family proteins expression. Somatic mutations in this pathway play important roles in the transduction of cell proliferation signals and may further promote genomic instability that results additional somatic mutations during cancer progression. RAS mutation is popular in FTC (about 50%), while RAF mutation is common in PTC (29-83%) (Henderson et al., 2009; Xing, 2005). Such an integrated model is depicted in Fig. 1.6 (Kondo et al., 2006).

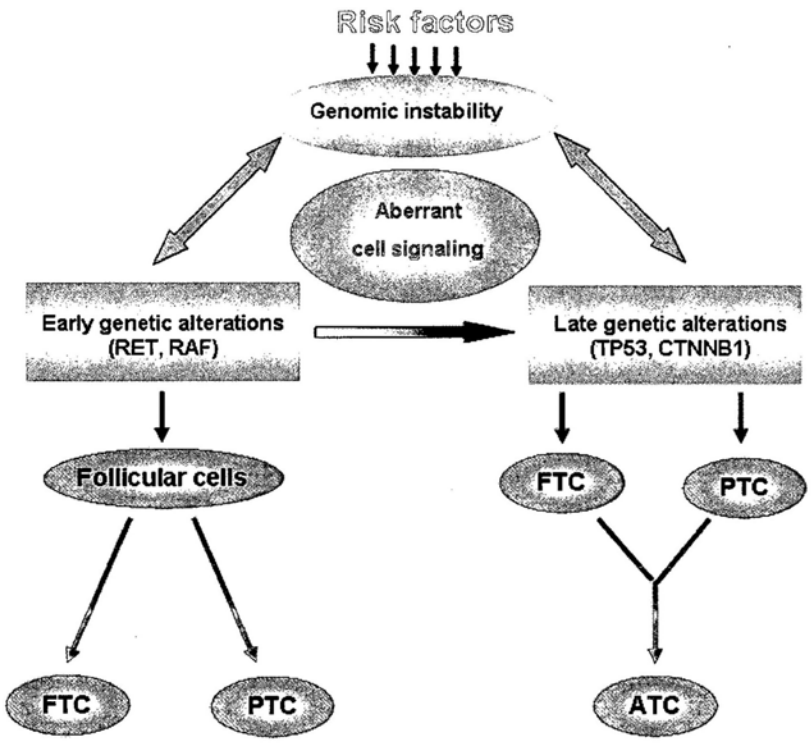


Fig. 1.6 The proposed model of thyroid carcinogenesis based on general concepts and specific pathways. On the basis of clinical, histological and molecular observations, risk factors such as exposure to radiation, induce genomic instability through direct and indirect mechanisms, resulting in early genetic alterations (RET, RAF, etc) that involve MAPKs signaling pathways. Oncogenic activation of MAPKs signaling further increases genomic instability and leads to later genetic alterations (TP53, CTNNB1) that involve other signaling pathways, cell-cycle regulators and various adhesion molecules. Accelerating the interactions between genomic instability and genetic alterations promotes progression from DTC to ATC (Kondo et al., 2006).

Besides the aberrant activation of MAPKs signaling pathways by RAS and/or RAF mutations, the tumor suppressor p53 is probably another prominent factor in eliciting cellular response to a variety of stress signals, including DNA damage, aberrant proliferative signals when inactivated. In unstressed cells, p53 protein is present at low levels and is relatively inactive. During the process of malignant transformation in human cells, a number of different changes occur in the cells that result in induction and activation of the p53 protein. In thyroid cancer, the p53 gene contains inactivating point mutations in approximately 55% of ATC, but relatively uncommon in PTC and FTC (Fagin et al., 1993; Ho et al., 1996; Ito et al., 1992). Thus, the loss of p53 activity provides a substantial advantage for cell transformation and uncontrolled proliferation (Chene, 2003; Strano et al., 2007; van Oijen and Slootweg, 2000).

1.3.4.2. Impairment of the balance between anti-apoptotic and pro-apoptotic proteins

Anti-apoptotic proteins of Bcl-2 family (Bcl-2 and Bcl-xL) are central to cell survival control in regulating apoptosis by modulating mitochondrial function (Danial, 2007). Abundant of examples exist where the anti-apoptotic proteins expression are highly expressed in cancers, such as lung cancer (Sanchez-Ceja et al., 2006), liver cancer (Watanabe et al., 2004), prostate cancer (Pollack et al., 2003), colorectal cancer (Biroccio et al., 2001), and etc. Apart from high levels of anti-apoptotic genes, cancers can acquire apoptosis resistance by down- regulation or mutation of pro-apoptotic genes. In certain types of cancers, the pro-apoptotic Bcl-2 family member Bax or Bad is mutated (Ionov et al., 2000; Lee et al., 2004). In thyroid cancer, higher levels of Bcl-2 or Bcl-xL

(Martinez-Brocca et al., 2008; Stassi et al., 2003), or lower levels of pro-apoptotic Bax (Cvejic et al., 2008), have been detected. It is widely accepted that anti-apoptotic proteins of Bcl-2 family can inhibit the activation of Bax or Bak (Cory and Adams, 2005). Overexpression of Bcl-2 or Bcl-xL was shown to prevent the conformation changes in Bax. The incorporation of Bax and Bak into large macromolecular complexes, and the competition of Bad between Bcl-2/Bcl-xL and Bax/Bak proteins, are all associated with cell apoptosis induction (Shore and Nguyen, 2008; Yip and Reed, 2008). Thus, the balance between pro- and anti-apoptotic proteins can determine cellular fate.

1.3.5 Apoptosis in thyroid cancer therapy

Defects in the apoptosis-inducing pathways not only can eventually lead to expansion of a population of tumor cells, but also can constitute important clinical problems such as chemoresistance in cancer therapy because chemotherapy and irradiation act primarily by inducing apoptosis. Apoptosis is a multi-step cell death programme in the body but apoptotic pathways defects in tumors are always exist, which make therapies in advanced thyroid cancer become more complicated. Therefore, how to induce tumor cells apoptosis and overcome apoptosis resistance are the main questions in cancer therapy.

In cancer treatment, both chemotherapy and irradiation kill target cells primarily by the induction of apoptosis. In thyroid cancer, radioactive iodine is widely used in DTC that has the ability to concentrate iodine. Cytotoxic chemotherapy is predominantly employed in ATC and may be used in the 20% of DTC that do not concentrate iodine (Pasieka, 2003). The commonly cytotoxic agents used in thyroid cancer therapy include:

DNA-damaging agents (doxorubicin, epirubicin), inhibitors of topoisomerases (etoposide), or antimetabolites (gemcitabine, methotrexate) and so on (Wartofsky and Van Nostrand, 2006). Apart from above, some new anticancer agents are found out. Recently, histone deacetylase (HDAC) inhibitor, such as sodium butyrate (NaB), has emerged as a promising new class of antitumor agent through the hyperacetylation of histiones and subsequent relaxation of chromatin, enhancing the cytotoxicity of drugs target DNA in cancer cells, including ATC (Catalano et al., 2006; Kim et al., 2003). No matter chemotherapy or irradiation, it has been evidenced that mitochondrial-mediated apoptosis is one of the main targeting apoptotic pathways (Galluzzi et al., 2006). For instance, chemotherapeutic agents cause DNA damage and lead to activation of pro-apoptotic protein Bax, or increase Bcl-2 and Bax ratio, and thus results Cyto C release and apoptosis *in vitro* and *in vivo* (Childs et al., 2002; McPake et al., 1998). However, few tumors are sensitive to these therapies, and the development of resistance to therapy is an important clinical problem. Accordingly, the molecular mechanisms in promoting or evading apoptosis may provide a novel opportunity for cancer drug development (Fulda and Debatin, 2004).

1.4 The aims of the study

Apoptosis plays important roles in thyroid cancer generation, development and therapy. Based on the epidemiological studies, iodine supplement influences thyroid cancer incidence and histologic types significantly. At the same time, the antitumor effect of iodine has been evidenced preliminarily but the underlying mechanism is unknown. The relations between iodine and thyroid cancer are very complicated and unknown, but

the process included in apoptosis may provide them clear clues: the aberrant signaling pathways and imbalance between pro- and anti-apoptotic proteins which activated by genetic alterations in thyroid cancer. Thus, this study will focus on these clues to explore the mechanism of iodine-induced apoptosis in thyroid cancer cells and the effects of iodine on chemotherapeutic agents-induced apoptosis. Various methods will be used to explore the following three issues:

- The underlying mechanisms of iodine-induced apoptosis in thyroid cancer cells.
- The effects and molecular mechanisms of iodine on chemotherapeutic agents-induced apoptosis in thyroid cancer cells.
- The mechanisms of MAPKs signaling pathways and the corresponding apoptosis regulators in iodine-induced apoptosis in thyroid cancer cells.

CHAPTER II: IODINE INDUCES APOPTOSIS THROUGH THE REGULATION OF MAPKS-RELATED P53, P21, AND BCL-XL IN THYROID CANCER CELLS

2.1 Introduction

Thyroid cancer, one of the most common endocrine malignancies, is mainly composed of three subtypes: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC). PTC and FTC are classified as differentiated thyroid cancer (DTC), while ATC is undifferentiated thyroid cancer (UDTC) (Braverman and Utiger, 2005; Jemal et al., 2008). Iodine plays a central role in thyroid physiology as it is a key element in the synthesis of thyroid hormones and maintains the normalcy of thyroid tissues. In thyroid cancer, previous researches have indicated that sufficient iodine supply prevents the transformation from DTC to ATC through DNA damage and gene mutagenesis in the development of thyroid cancer (Dal Maso et al., 2009; Dijkstra et al., 2007; Krohn et al., 2007; Maier et al., 2007). Moreover, the mutant genes (such as BRAF, RAS, and so on) always activate aberrant cell signaling pathways that control cell proliferation and apoptosis, including mitogen activated protein kinases (MAPKs) (Liu et al., 2008). Moreover, radioactive ^{131}I therapy is a major ancillary tool that benefits the prognosis of thyroid cancer significantly (Toniato et al., 2008). Recently, studies have demonstrated that iodide (I^-) could induce apoptosis in thyroid cells *in vitro* via producing excess molecular iodine (I_2) with aid of endogenous peroxidases (Smyth, 2003; Vitale et al., 2000). Subsequent studies have indicated that iodine could prevent cancer development *in vivo* and induce apoptosis *in vitro* (Garcia-Solis et al., 2005; Shrivastava

et al., 2006). Therefore, iodine may benefit thyroid cancer by preventing tumor transformation and inducing the death of tumor cells, probably in the form of apoptosis.

MAPKs signaling pathways act critical roles in cell proliferation and apoptosis because they can function as a pro- or anti-apoptotic factor by switching to turn on or off the substrate proteins, including transcription factor p53 and apoptosis regulators Bcl-2 family proteins (Osaki et al., 2004; Wada and Penninger, 2004). Moreover, various molecules related to iodide metabolism are modulated by MAPKs pathways directly or indirectly. For instance, the activation of ERK signaling pathway modulates the level and the localization of sodium iodide symporter (NIS), which is one main protein in mediating active influx of iodide into thyrocytes (Jung et al., 2008). However, little is known about the role of these pathways in iodine-induced apoptotic effects in thyroid cancer cells. The roles of MAPKs pathways in apoptosis and iodide metabolism led us to hypothesize that these pathways might be involved in iodine-induced apoptosis.

Therefore, it is of interest to investigate iodine-induced effects in thyroid cancer cells and elucidate the molecular mechanisms involved in the process. This section will focus on apoptotic effects of iodine in thyroid cancer cells, and elucidate the underlying mechanisms that contribute to the apoptotic effect of iodine.

2.2 Materials and methods

2.2.1 Cell culture

Human anaplastic ARO, papillary NPA and follicular WRO thyroid cancer cell lines were used in the study. The three cell lines all harbor mutant p53, R273H in ARO, G266E in NPA and P233L in WRO respectively (Nagayama et al., 2000). Cells were maintained in RPMI 1640, supplemented with 1% L-glutamine, and 100 units/ml penicillin-100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37°C and 5% CO_2. Fresh medium was always added to the cells the day before experiments, and the cells were separated via trypsinization using 0.05% trypsin/0.53 mM EDTA. All reagents used in the cell culture were purchased from Gibco (Grand Island, NY).

2.2.2 Reagents and antibodies

Primary antibodies against $p21^{Waf1/Cip1}$ (12D1), Bak, phospho-p44/42 MAPK(ERK1/2) (Thr202/Tyr204), p44/42 MAPK(ERK1/2) and phospho-p38 MAPK (Thr180/Tyr182) (28B10) were obtained from Cell Signaling Technology (Beverly, MA). The other primary antibodies against cytochrome c, Bcl-xL (7B2.5), p53 (DO-1), caspase 3 (H-277), JNK (F-3), phospho-JNK (Thr183/Tyr185) (G-7), p38 (A-12) and β-Actin (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horse radish

peroxidase (HRP) labelled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Iodine solution (I_2, 50mM) was purchased from Sigma-Aldrich (St Louis, MO). ERK inhibitor PD98059 (PD) and p38 inhibitor SB203580 (SB) were obtained from Promega (Madison, WI). JNK inhibitor SP600125 (SP) was purchased from Sigma-Aldrich (St Louis, MO).

2.2.3 Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instruction (Sigma-Aldrich, MO). Briefly, cells were seeded at 1×10^4 cells/well in 96-well plates for 24 h before treatment followed by incubation with different concentrations of iodine for indicated time points. After adding 100 μl/well MTT solution (0.5 mg/ml in PBS), the cells were incubated for another 4 h. Supernatants were then removed and the formazan crystals were solubilized in 100 μl/well DMSO. The absorbance at 570 nm/630 nm of each sample was measured using multilabel plate reader (PerkinElmer, MA). Every experiment was repeated at least 3 times.

2.2.4 Apoptosis analysis

Apoptosis assay was performed using APO-DIRECTTM TUNEL ASSAY kit (Chemicon international, CA). Cells were harvested and fixed in 1% paraformaldehyde for 60 min at 4°C, followed by a second fixation in 70% (v/v) ethanol overnight at -20°C. Then the cells were treated with various reagents for a designed period according to the manufacturer's instruction. Finally, the cells were analyzed by flow cytometry using FACS Vantage machine (Becton Dickinson, NJ). The CellQuest software (Verity Software House, USA) were used to analyze the data.

2.2.5 Measurement of mitochondrial membrane potential $\Delta\psi m$ by flow cytometry

Forty nM of DioC6 (Sigma-Aldrich, MO) were incubated with treated cells at indicated time points for 15 min at 37°C. The harvested cells were washed with ice-cold PBS and analyzed by flow cytometry using Becton Dickinson FACS Vantage machine (Becton Dickinson, NJ). Cells with low $\Delta\psi_m$ were presented as a percentage of the total cell population. The CellQuest software (Verity Software House, USA) were used to analyze the data.

2.2.6 Western blot analysis

Total protein was extracted by RIPA buffer [1×PBS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), pH 7.6 with protease

inhibitors 0.1% aprotonin, 100 μM sodium orthovanadate and 100 μg/ml phenylmethy-sulphonyl-fluoride (PMSF)]. Cytosolic protein fraction was isolated according to a previous procedure (Li et al., 2006). Briefly, the treated cells were harvested via trypsinization, then washed with ice-cold PBS and extracted with cold homogenization buffer (20 mM HEPES, 20 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM Na3VO3, pH 7.5, and 1×Protease Inhibitor Cocktail). The re-suspended cells were homogenized with 10 strokes of Teflon homogenizer for 10 min at 4°C and the supernatants were further 10,000 g for 3 min at 4°C to collect the supernatants (the cytosolic fraction). Protein concentration was determined by Bio-Rad Dc reagent (Bio-Rad, CA). Ten μg of the cytosolic protein or 30 μg total protein was separated on 8%-15% SDS/polyacrylamide gels and then transferred onto a nitrocellulose membrane (Amersham Biosciences, NJ). The membrane was blocked with 5% nonfat milk and then probed with specific primary antibody overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature after washing. The signals were visualized with ECL Kit (GE healthcare, UK). All western blot analysis was performed at least twice independently.

2.2.7 RNA interference

Thyroid cancer cells ARO (1×10^5 cells/well) were seeded in six-well plates and incubated in 2 ml of normal growth medium containing serum without antibiotics for 24 h. Briefly, for each well of cells to be transfected, 1.5 μg of the p21 siRNA (Cell

Signaling, MA) and Bcl-xL siRNA (Invitrogen, CA) were diluted with 100 μl of Opti-MEM I Reduced Serum Medium (Invitrogen, CA), and then 3 μl of Lipofectamine™ RNAiMAX (Invitrogen, CA) was diluted with 100 μl of Opti-MEM I Reduced Serum Medium (Invitrogen, CA) as manufacturer recommendation. A non-targeting siRNA (Invitrogen, CA) was used as negative control. After 6 h incubation at 37°C, the medium was replaced with fresh growth medium containing serum and the cells were incubated for another 18 h before use. Iodine (100 μM) was then added and incubated for 24 h. The cells were harvested for western blot analysis or apoptosis analysis.

2.2.8 Statistics analysis

Differences between groups were examined for statistical significance using Student's t-test. All P-values are two-sided and $P<0.05$ was considered statistically significant. All statistical calculations were performed by the SPSS software (version 13.0, IL). The data were presented as mean ± SD from at least three independent experiments.

2.3 Results

2.3.1 Iodine induces apoptosis in thyroid cancer cells

To investigate the effects of iodine on thyroid cancer cells, the growth inhibitory effect of iodine was first assessed in three human thyroid cancer cell lines, ARO, NPA and WRO. Cell viability was determined by MTT assay after 24 h, 48 h and 72 h iodine treatment at various concentrations (0-100 μM). In ARO, NPA and WRO cells, growth inhibition was induced in a dose- and time-dependent manner (Fig. 2.1A).

To further determine whether the cell death induced by iodine was associated with apoptosis, the apoptotic cells were detected with TUNEL assay. After the 24 h treatment with various concentrations of iodine (0-100 μM), occurrence of apoptosis upon iodine administration was observed in ARO, NPA and WRO cells in a dose-dependent manner (Fig. 2.1B). However, the sensitivities of three thyroid cancer cell lines to iodine were quite different. For instance, at the medium dose of iodine (75 μM), the apoptotic rates of ARO and WRO were similar, 7.39 ± 1.65% and 8.35 ± 4.58% respectively, which were much lower than in NPA cells, 43.25 ± 8.03% ($P<0.01$). Whereas, at the high dose of iodine (100 μM), the apoptotic rates in three cell lines were all markedly up-regulated, but the apoptotic rate of ARO (31.57 ± 9.56%) was still much lower than that of NPA (81.22 ± 6.21%) ($P<0.01$) and WRO (56.53 ± 8.68%) ($P<0.05$).

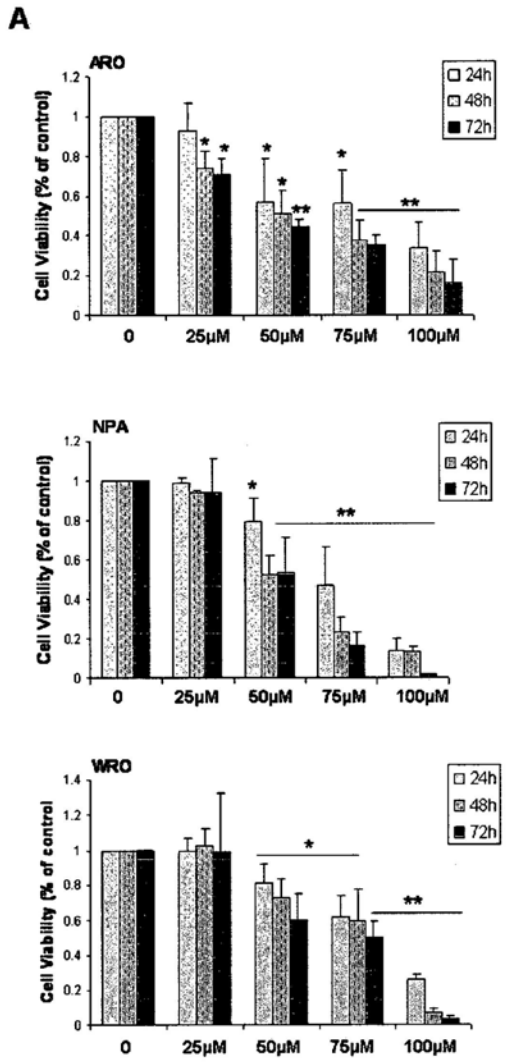


Fig. 2.1 A. Iodine inhibits cell proliferation in thyroid cancer cells. MTT assay was used to determine the cell viability. ARO, NPA and WRO were treated with various concentrations (0-100 μM) of iodine for 24 h, 48 h and 72 h respectively. The percentage of cell viability was summarized in the histograms. The data represent mean ± SD of three independent experiments. *P<0.05 and **P<0.01, versus the control group.

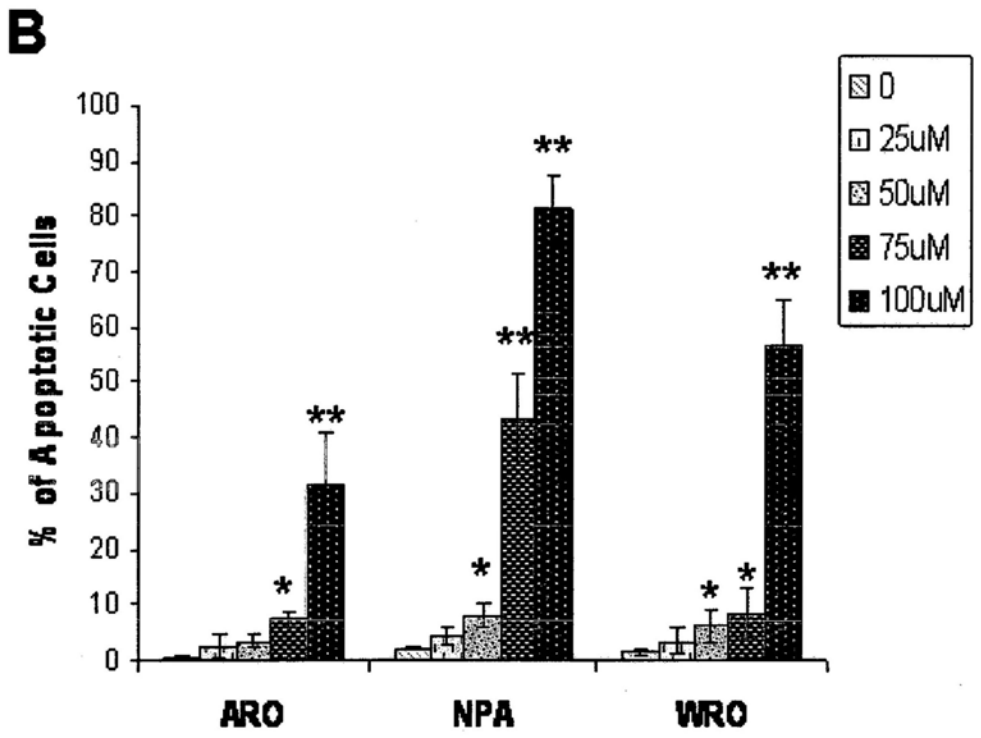
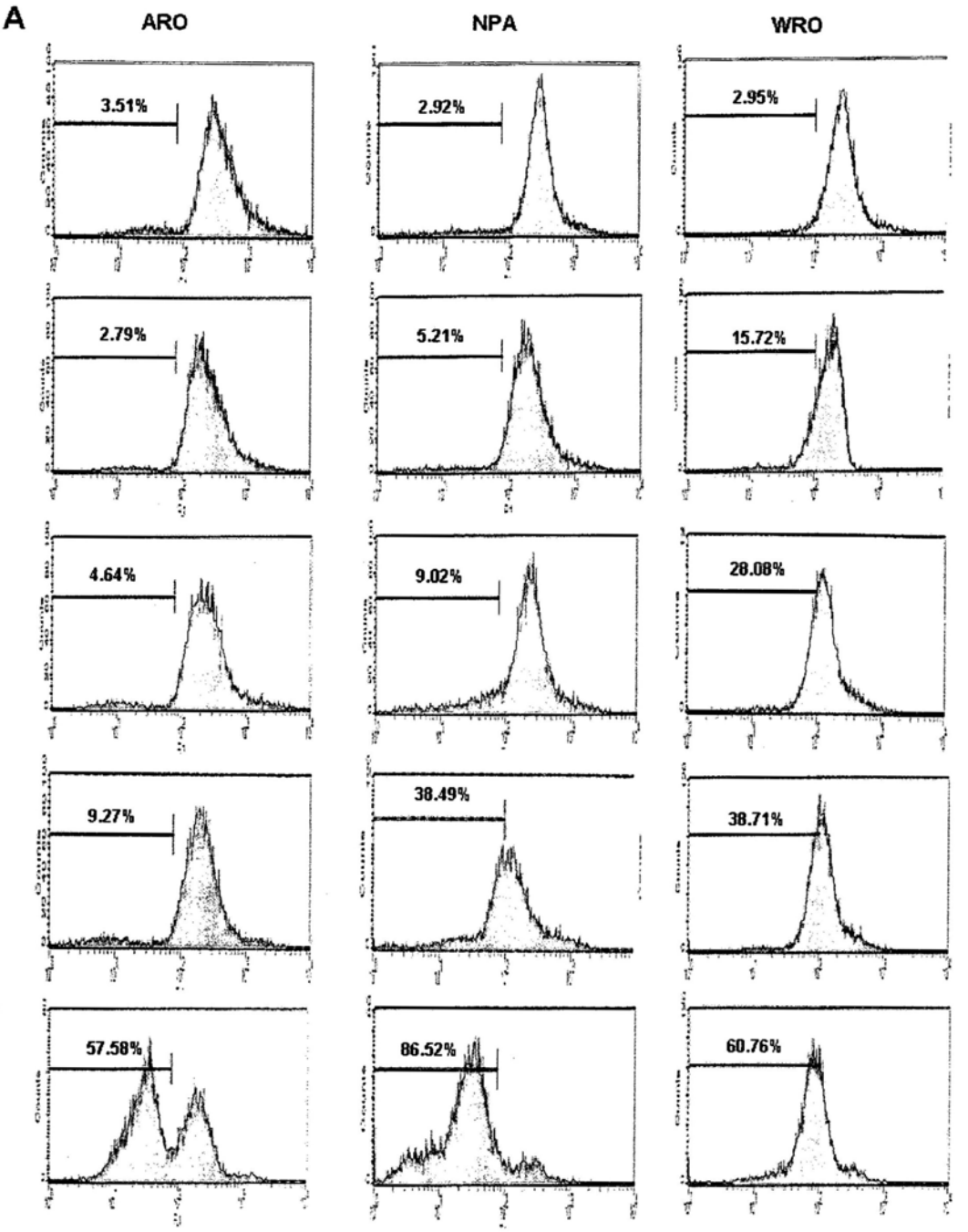


Fig. 2.1 B. Iodine induces apoptosis in thyroid cancer cells. TUNEL assay was used to detect apoptosis by flow cytometry. ARO, NPA and WRO were treated with various concentrations (0-100 μM) of iodine for 24 h. The percentage of apoptotic cells was summarized in the histograms. The data represent mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01, versus the control group.

2.3.2 Mitochondrial death pathway is involved in iodine-induced apoptosis in thyroid cancer cells

To examine whether the apoptosis induced by iodine was through the mitochondrial death pathway, the dose course of mitochondrial membrane potential (MMP, $\Delta\psi_m$) collapse was examined in iodine-treated thyroid cancer cells. Iodine caused a significant reduction of $\Delta\psi_m$ in all three types of tumor cells in a dose-dependent manner (Fig. 2.2A and 2.2B). It was noted that sensitivities of the cells to iodine were different among these three types of cells. NPA and WRO cells were much more sensitive to iodine than ARO cells. As shown in Fig. 2.2B, the reduction of $\Delta\psi_m$ at the medium dose of iodine (75 μM) in ARO cells (9.25 ± 4.48%) was obviously less than in NPA (33.03 ± 5.49%) ($P<0.01$) and in WRO cells (28.71 ± 10.63%) ($P<0.05$) respectively. The similar result could be found at the high dose of iodine (100 μM), the reduction of $\Delta\psi_m$ in ARO was 48.72 ± 7.84% which was markedly lower than NPA 88.17 ± 4.58% ($P<0.01$) and WRO 62.11 ± 3.07% ($P<0.05$). The further examination was conducted on the effects of iodine on the mitochondrial pathway by analysis of Cyto C release, which contributes to caspase 3 activation. As shown in Fig. 2.2C, after the 24 h treatment with various concentrations of iodine (0-100 μM), increased Bak and cytosolic Cyto C proteins were observed, especially at high dose of iodine (100 μM). Additional observation showed that procaspase 3 was markedly decreased in a dose-dependent manner, particularly in NPA and WRO cells (Fig. 2.2C).


A
ARO
NPA
WRO
3.51%
2.92%
2.95%
2.79%
5.21%
15.72%
4.64%
9.02%
28.08%
9.27%
38.49%
38.71%
57.58%
86.52%
60.76%

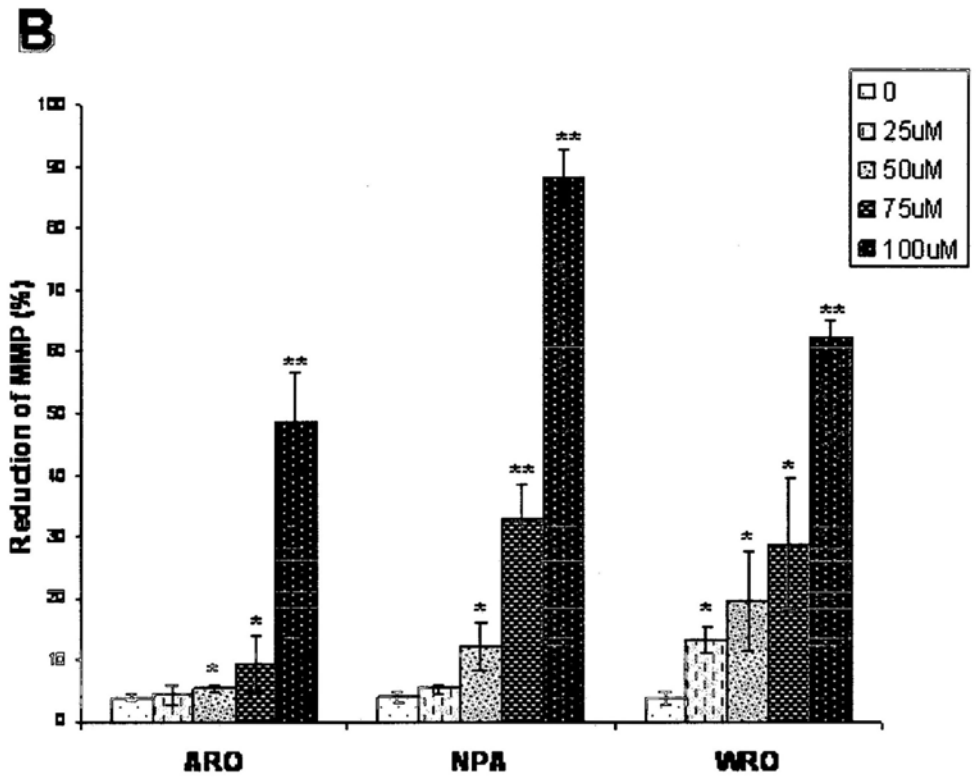


Fig. 2.2 A and B. Mitochondrial membrane collapse in iodine-induced apoptosis in thyroid cancer cells. The mitochondrial membrane potential collapse ($\Delta\psi m$) was measured by flow cytometry after staining the cells with DioC6 and quantitative analysis of $\Delta\psi m$ was shown. ARO, NPA and WRO were treated with various concentrations (0-100 μM) of iodine for 24 h. The data represent mean ± SD of three independent experiments. *P<0.05 and **P<0.01, versus the control group.

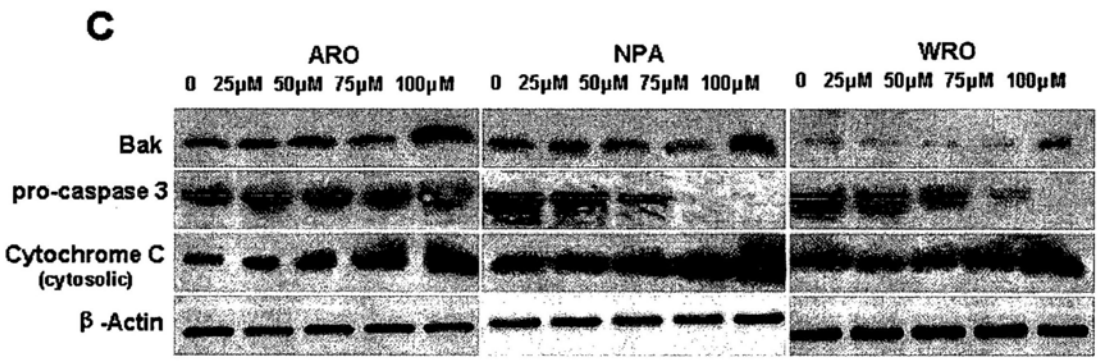


Fig. 2.2 C. Mitochondrial death pathway is involved in iodine-induced apoptosis in thyroid cancer cells. Western blot analysis was used to detect the expression of Bak, procaspase-3 and Cyto C (cytosolic fraction). ARO, NPA and WRO were treated with various concentrations (0-100 μM) of iodine for 24 h and the protein was isolated for Western blot analysis. β-Actin in the total protein was used as a control and experiments were repeated at least twice.

2.3.3 Iodine induces p21 accumulation in p53 mutant thyroid cancer cells

To determine whether the apoptosis induced by iodine was correlated with mutant p53 and p21 in thyroid cancer cells, the expression of p53 and p21 was evaluated by Western blot analysis. The results showed that iodine reduced the level of mutant p53 but increased the expression of p21 in a dose- and time-dependent manner in three thyroid cancer cell lines (Fig. 2.3A and 2.3B). p21 siRNA was used to knockdown the p21 expression and investigate its role in thyroid cancer cells treated by iodine. Knockdown of p21 was performed in ARO cells followed by 100 μM of iodine treatment for 24 h. As shown in Fig. 2.3C, knockdown of p21 was confirmed by Western blot analysis. In the following step, the investigations were carried out on the changes in apoptosis after the application of p21 siRNA. Compared with the negative control (cells transfected with non-targeting siRNA), p21 siRNA markedly down-regulated apoptosis of ARO cells at 24 h after the p21 siRNA transfection (35.29 ± 7.66% *vs.* 19.68 ± 4.07%, $P<0.05$) (Fig. 2.3D).

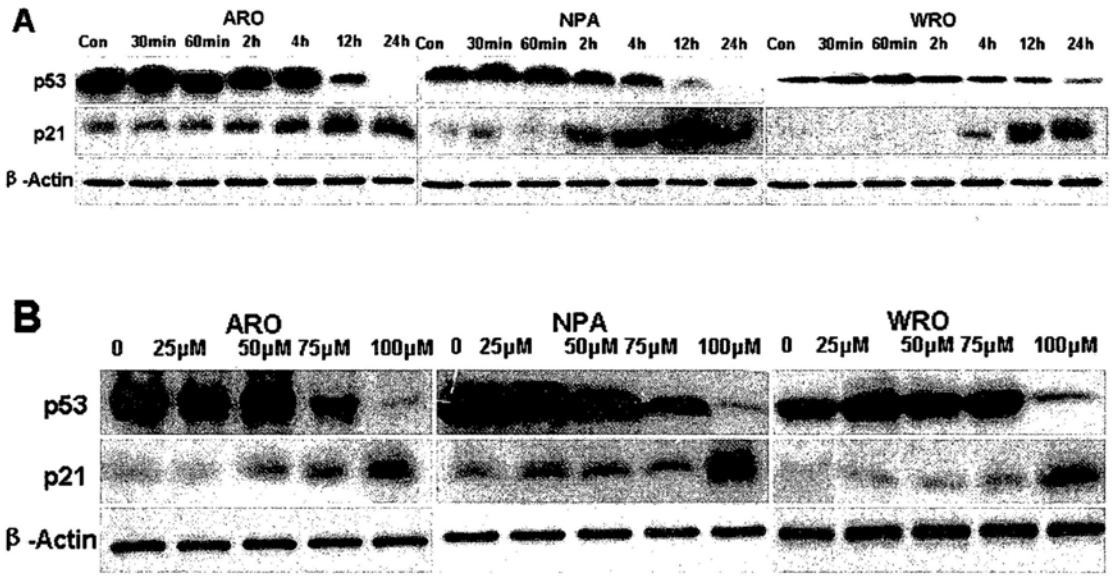


Fig. 2.3 A and B. Time- and dose-course experiments of iodine-mediated apoptosis requires mutant p53 down-regulation and p21 accumulation in thyroid cancer cells. **A: Time-course experiment.** ARO, NPA and WRO cells were treated with 100 μM iodine for the indicated time points (30 min to 24 h). After the treatment, Western blot was used to detect the expression of p53 and p21. β-Actin in the total protein was used as a control and experiments were repeated at least twice. **B: Dose-course experiment.** ARO, NPA and WRO cells were treated with different concentrations of iodine (0-100 μM) for 24 h. After the treatment, Western blot was used to detect the expression of p53 and p21. β-Actin in the total protein was used as a control and experiments were repeated at least twice.

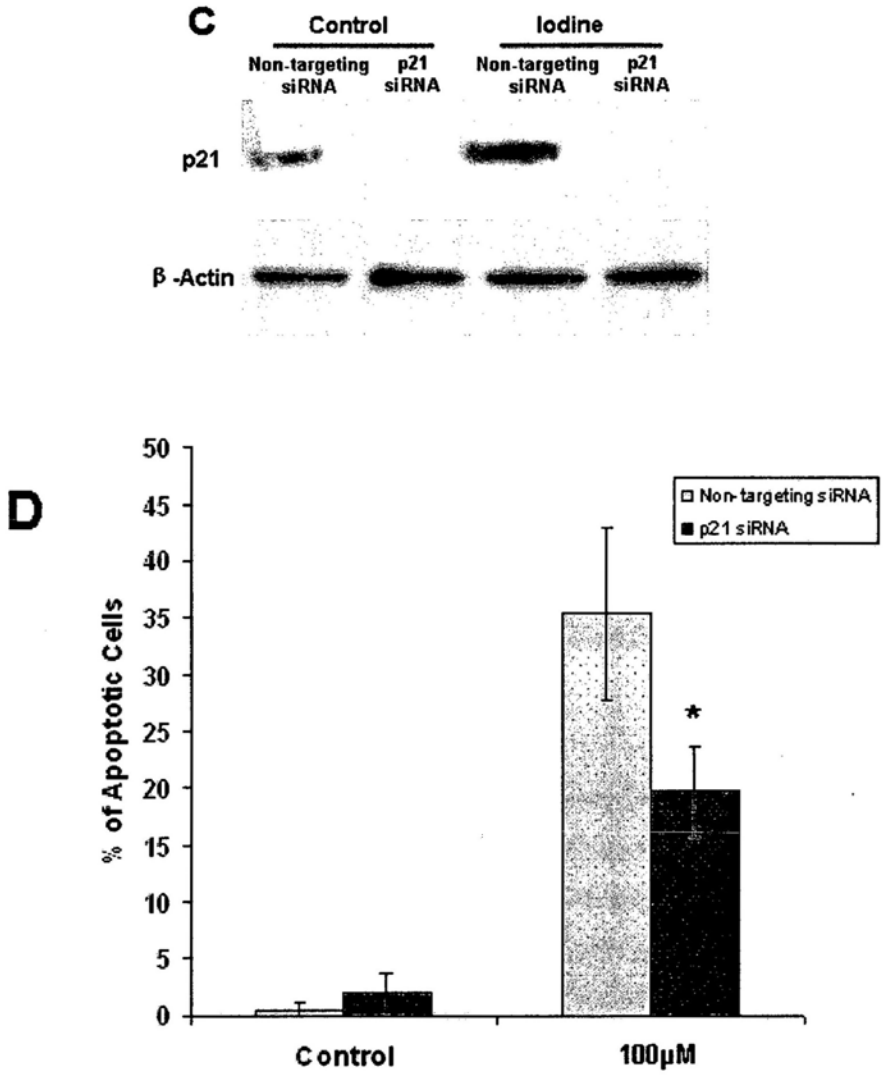


Fig. 2.3 C and D. Effects of p21 knock down in ARO cells treated by iodine. C: p21 siRNA effectively knocked out the expression of p21 protein in ARO cells as detected by Western blot. Cells transfected with non-targeting siRNA was used as negative control. **D:** ARO cells were transfected with p21 siRNA or non-targeting siRNA for 24 h, and then treated with 100 μM iodine for 24 h. The percentage of apoptotic cells was

determined by flow cytometry and the result was summarized in the histograms. The data represent mean ± SD of three independent experiments. *P<0.05, versus ARO cells treated by iodine in non-targeting siRNA transfected group.

2.3.4 Up-regulation of Bcl-xL expression protects thyroid cancer cells from iodine-induced apoptosis

To test whether anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, were involved in the regulation of apoptosis induced by iodine, the levels of Bcl-2 and Bcl-xL by Western blot analysis were checked and the results were shown as Fig. 2.4A and 2.4B. From the figures it could be found that the expression of Bcl-xL protein was significantly up-regulated, especially at the high dose of iodine (100 μM) (Fig. 2.4A and 2.4B), but no significant difference in Bcl-2 protein expression was found (data not shown). To clarify the function of Bcl-xL, the knockdown of Bcl-xL expression by specific Bcl-xL siRNA was performed (Fig. 2.4C). Bcl-xL siRNA significantly increased apoptotic cells of ARO from 35.29 ± 7.66% to 82.29 ± 7.59% (P<0.01, Fig. 2.4D)

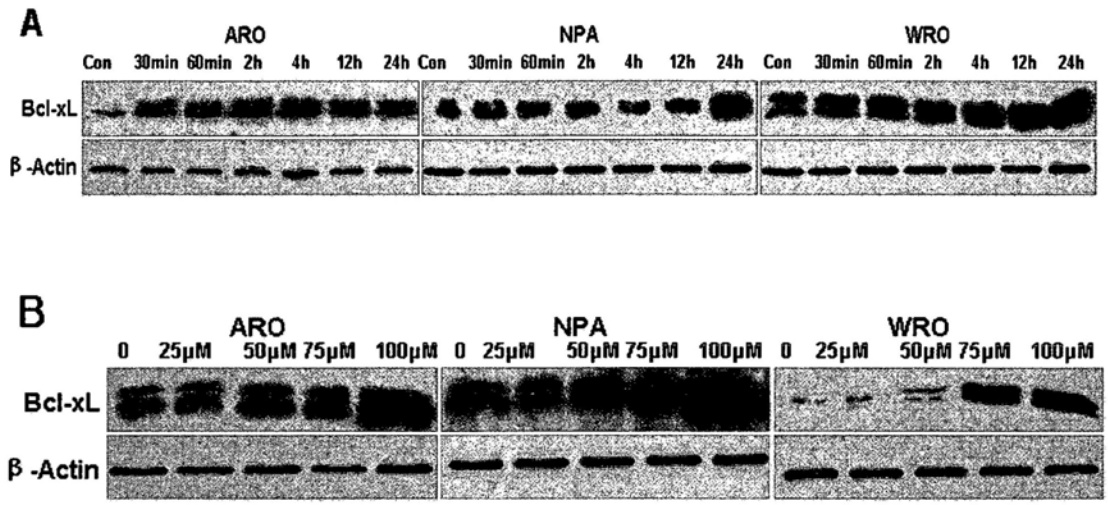
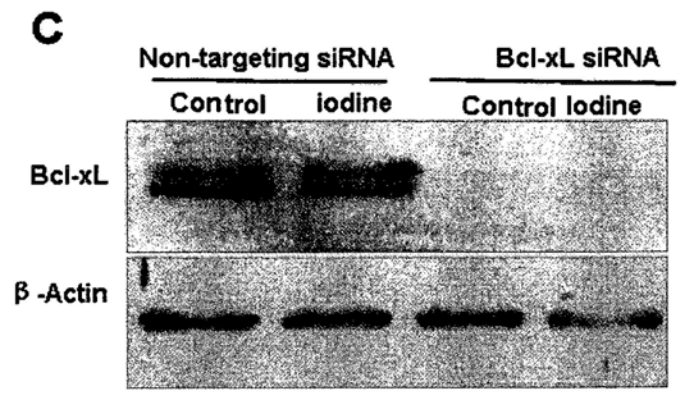


Fig. 2.4 A and B. Up-regulation of Bcl-xL expression protects thyroid cancer cells from iodine-induced apoptosis. A: Time-course experiment. ARO, NPA and WRO cells were treated with 100 μM iodine for the indicated time points (30 min to 24 h). After the treatment, Western blot was used to detect the expression of Bcl-xL. **B: Dose-course experiment.** ARO, NPA and WRO cells were treated with different concentrations of iodine (0-100 μM) for 24 h. After the treatment, Western blot was used to detect the expression of Bcl-xL. β-Actin in the total protein was used as a control and experiments were repeated at least twice.



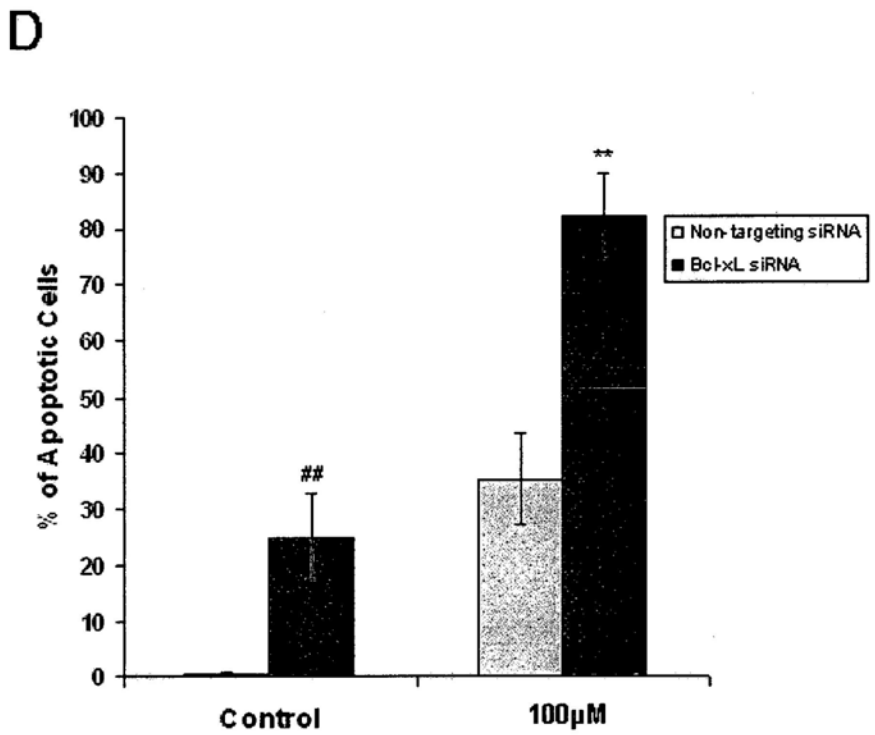


Fig. 2.4 C and D. Effects of Bcl-xL knock down in ARO cells treated by iodine. C: Western blot was used to confirm that the expression of Bcl-xL was effectively blocked by Bcl-xL siRNA in ARO cells. Cells transfected with non-targeting siRNA was used as

negative control. **D:** ARO cells were transfected with Bcl-xL siRNA or non-targeting siRNA for 24 h, and then treated with 100 μM iodine. Apoptotic cells were determined by flow cytometry after staining the cells with TUNEL. The percentage of the apoptotic cells was summarized in the histograms. The data represent mean ± SD of three independent experiments. $^{\#\#}P<0.01$, versus non-targeting siRNA transected cells in control group; $^{**}P<0.01$, versus non-targeting siRNA transfected cells in iodine-treated group.

2.3.5 MAPKs pathways are activated in iodine-induced apoptosis

To explore whether MAPKs pathways are involved in iodine treated thyroid cancer, the expression of the distinct subfamily members of MAPKs (ERK1/2, p38 and JNK1/2) were detected after exposure of thyroid cancer cells to 100 μM of iodine for a period of 30 min to 24 h by Western blot. The results showed that the levels of the phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 (p-p38), and phosphorylated JNK1 (p46) (p-JNK1) were transiently up-regulated in ARO, NPA and WRO cells (Fig. 2.5A). Meanwhile, the activation of these cascades started at 30 min or 60 min, and most of them peaked at 4 h. To understand more about the activation of these cascades on the induction of apoptosis, the specific inhibitors were used. ARO cells were pretreated with 40 μM PD, 40 μM SB, or 50 μM SP for 1 h respectively, before 100 μM of iodine were added. After adding iodine, the cells were cultured for another 12 h. The result showed

that the cell viability of ARO cells was markedly decreased by all three inhibitors ($P<0.05$) (Fig. 2.5B).

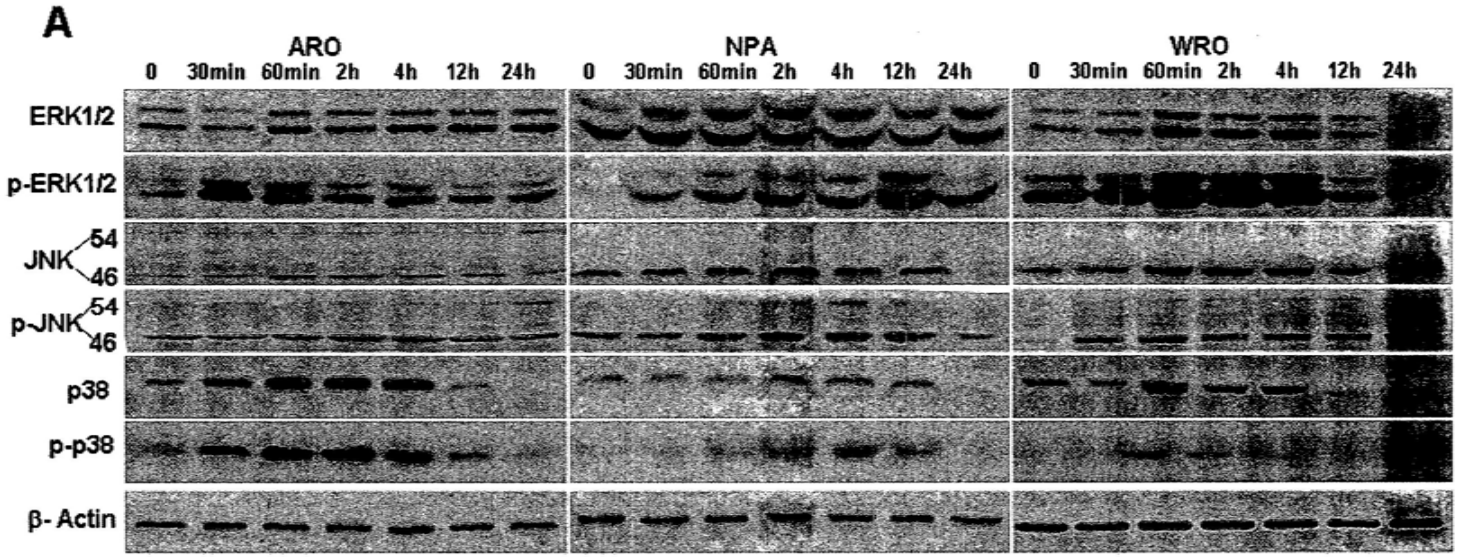


Fig. 2.5 A. Activation of MAPKs pathways in iodine-treated thyroid cancer cells. ARO, NPA and WRO cells were treated with 100 μM of iodine for the indicated time points (30 min to 24 h). Western blot analysis was used to detect the expression of phophorylated and total molecules of ERK1/2, p38, and JNK1/2. β-Actin in the total protein was used as a control and experiments were repeated at least two times.

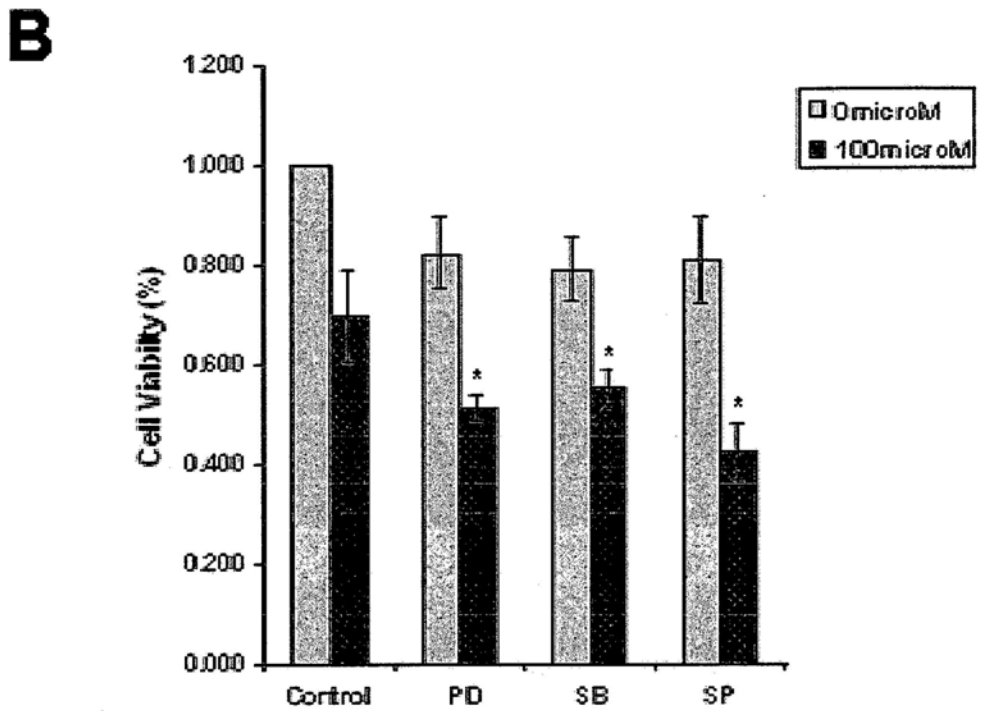


Fig. 2.5 B. Effects of ERK, p38 and JNK inhibitors on the viability of iodine-treated ARO cells. MTT assay was used to analyze the cell viability. ARO cells were pretreated with 40 μM PD98059 (PD), 40 μM SB203580 (SB), or 50 μM SP600125 (SP) for 1 h and then treated with 100 μM iodine for 12 h. The percentage of cell viability of treated cells was assayed by MTT. Experiments were repeated three times. The data were expressed as mean ± SD. *P<0.05, versus iodine treatment alone.

2.3.6 Iodine modulates the expressions of p53, p21 and Bcl-xL through MAPKs signaling pathways

To study how the activation of signal cascades MAPKs pathways functions in the reduction of p53, accumulation of p21 and Bcl-xL in iodine-treated thyroid cancer cells, ARO cells were treated with 100 μM iodine for 12 h in the presence or absence of the specific inhibitors. As shown in Fig. 2.5C, in iodine-treated ARO cells, mutant p53 expression was down-regulated by all three inhibitors, and the level of p21 also was reduced by these inhibitors (PD and SB) except the JNK1/2 inhibitor (SP). Meanwhile, PD and SB promoted Bcl-xL expression while SP inhibited it.

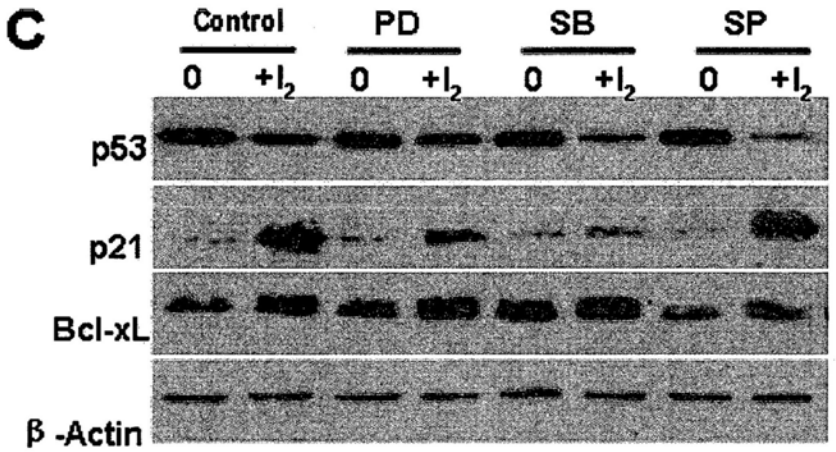


Fig. 2.5 C. Effects of ERK, p38 and JNK inhibitors on p53, p21 and Bcl-xL expressions in iodine-treated ARO cells. Western blotting analysis was used to detect the expressions of p53, p21 and Bcl-xL. ARO cells were treated as in Fig. 2.5B. Control cells were treated with vehicle. β-Actin in the total protein was used as a control and experiments were repeated at least twice.

2.4 Discussion

Iodine is well known for its function in thyroid gland to produce thyroid hormones, but is scarcely known about its correlations with thyroid cancer at the molecular level. Epidemiological studies have shown that sufficient iodine supply can prevent the development of thyroid cancer (Dal Maso et al., 2009; Dijkstra et al., 2007; Krohn et al., 2007; Maier et al., 2007). However, the mechanism is unclear. Previous studies have shown that the function or expression of iodide-specific transportation genes such as NIS is usually impaired in thyroid cancer cells including these used in the present study (Schlumberger et al., 2007), which will destroy iodide (I^-) uptake. Whereas, it has been reported that excess molecular iodine (iodine, I_2) generation and oxidation of ionic iodine by endogenous peroxidases are key steps in iodide-induced apoptosis (Vitale et al., 2000), and that iodine intake can be independent of the iodide-specific transportation genes such as NIS (Garcia-Solis et al., 2005; Shrivastava et al., 2006). Therefore, molecular iodine (iodine, I_2) was used in the present experiment to exert its effects.

Apoptosis induced by iodine in thyroid cancer cells appears to be via the mitochondrial pathway since iodine targets the mitochondria to reduce mitochondrial membrane potential, increase the mitochondrial-related pro-apoptotic molecule Bak, and promote the release of Cyto C from the mitochondria. It is interesting to note that the three types of thyroid cancer cell lines have different sensitivities to iodine-induced apoptosis. The apoptotic rate of PTC cell line NPA appears to be much higher than that of ATC cell line ARO and FTC cell line WRO. This finding may suggest that iodine-based therapy is more suitable for PTC or the insufficient iodine intake may affect

PTC more than other types of thyroid cancers. In line with this finding, an epidemiological study shows that iodine deficiency increases the incidence of PTC, resulting in the decreased ratio of FTC to PTC (Burgess et al., 2000). Therefore, these data support the epidemiological or clinical observation that the state of iodine can influence the distribution of histological subtypes of thyroid cancer (Dijkstra et al., 2007)

Further experiments reveal that iodine-induced apoptosis is associated with p53 and p21 expression. Iodine could reduce the expression of p53 protein but increase the level of p21 in all three types of thyroid cancer cells tested. The effect of iodine on these two molecules is in both time- and dose-dependent manners. p53 acts as a major defense against cancer, but malfunction of the p53 pathway is an almost universal hallmark of cancer. p53 is dysfunctional in approximately 55% of ATC (Smallridge et al., 2009; Whibley et al., 2009). It is known that ARO harbors p53 R273H mutant, NPA and WRO contain p53 G266E and P223L mutants respectively (Nagayama et al., 2000). Among these three p53 mutants, R273H is known to exhibit a dominant negative effect through inactivation of the function of wild-type p53 or gains new function as an anti-apoptotic agent to induce drug resistance (Willis et al., 2004; Wong et al., 2007). Therefore, the down-regulation of R273H p53 by iodine in ARO cells should facilitate apoptosis rather than inhibit it, which benefits the anti-tumor treatment. It is unknown whether p53 G226E in NPA and p53 P223L in WRO can also function as R273H p53 in ARO. However, at least it is known that thyroid cancer cells with p53 G226E and p53 P223L do not possess the function of wild-type p53 (Xiao et al., 2009). Notably, In thyroid cancer, the p53 gene contains inactivating point mutations in approximately 55% of ATC, but relatively uncommon in DTC, suggesting that p53 mutations play a crucial role in the progression

of DTC to ATC (Fagin et al., 1993; Ho et al., 1996; Ito et al., 1992). Our results provide molecular evidence that iodine functions crucially in the biologically aggressive subtypes of thyroid cancer through the mutant form of p53 protein. p21 is deemed to be an important molecule in tumor development and drug-induced tumor suppression (Gartel and Tyner, 2002). This notion is supported in this study since the block of p21 significantly diminishes iodine-mediated apoptosis. In fact, the up-regulation of p21 has been reported to enhance apoptosis induced by antitumor agents in thyroid cancer cells (Catalano et al., 2009; Xiao et al., 2009; Yang et al., 2003). Although p21 can be induced by both p53-dependent and p53-independent mechanisms, in the present study p53 is unlikely to promote the iodine-induced p21 since the level of p53 is reversely associated with p21 and p53 in the cells tested is mutant. Taken together, these data provide evidence that iodine can induce apoptosis by induction of p21 and/or reduction of mutant p53 in human thyroid cancer cells.

Surprisingly, high doses of iodine enhance the level of Bcl-xL rather than inhibit it. Bcl-xL is a well known anti-apoptotic protein and it may influence the anti-tumor effect of iodine. Previous studies have shown that Bcl-xL up-regulation is one main pathway that results in chemoresistance (Wang et al., 2009), whereas predominant Bcl-xL knockdown or Bcl-xL inhibitors overcome chemoresistance (Bai et al., 2005; Shoemaker et al., 2006). The findings indicate that the thyroid tumor cells, in response to the iodine-mediated damage, express more Bcl-xL as a compensated measurement to protect themselves. Based on this concept, once this compensated Bcl-xL is blocked, iodine should induce more apoptosis in thyroid cancer cells. Indeed, this is the case in our experiment. The percentage of apoptotic thyroid cancer cells is significantly increased when the cells

are treated with iodine in the presence of Bcl-xL siRNA. In the other aspect, it is evident that Bcl-xL can expedite cell differentiation (Liste et al., 2007). Data have showed that the level of Bcl-xL is higher in DTC than in ATC (Martinez-Brocca et al., 2008; Stassi et al., 2003). Although in this study the basic level of Bcl-xL in ATC was similar to that in NPA and WRO, both of which are DTC, iodine-induced Bcl-xL was much higher in the latter than in the former after 24 h-treatment. This finding suggests that iodine may function in favor of thyroid tumor differentiation. Therefore, based on the known information and our results, it can be concluded that Bcl-xL may be one of the critical proteins for iodine to influence the development of thyroid cancer, which may open up a novel approach to thyroid cancer therapy.

The expressions of p53, p21 and Bcl-xL are closely associated with MAPKs signaling pathways, which function critically in the regulation of cell proliferation and apoptosis (Franke et al., 2003; Johnson and Lapadat, 2002). For instance, p53-mediated apoptosis is closely correlated with ERK, or p38 activation (She et al., 2001). Using relevant inhibitors of these kinases, this study indicated that all tested subfamily members of MAPKs function as anti-apoptotic factors in thyroid tumor cells studied. The anti-apoptotic feature of these 3 kinases tested was reinforced by the experiment showing that the inhibition of ERK1/2 and p38 reduced the viability of thyroid tumor cells and was negatively associated with the levels of anti-apoptotic R273H-mutated p53 and pro-apoptotic p21. These findings suggest that the activation of ERK1/2 and p38 function as anti-apoptotic factors likely through modulating R273H-mutated p53 expression. Meanwhile, the inhibition of JNK pathway was negatively associated with the levels of R273H-mutated p53 and Bcl-xL but not correlated with p21 expression, suggesting that

JNK functions as an anti-apoptotic protein factor not only through modulating R273H-mutated p53 expression but also Bcl-xL expression. Collectively, the modulation of the expression of R273H-mutated p53 is likely a main step for ERK1/2, p38 and JNK to function as anti-apoptotic factors in iodine-induced apoptosis, and targeting Bcl-xL is another pathway for JNK to function as an anti-apoptotic factor. Therefore, the findings reveal that iodine plays an important role in apoptosis of thyroid cancer via regulation of three major kinases, which further influence the levels of mutant p53, Bcl-xL and p21.

In conclusion, our results demonstrate that the apoptotic effects of iodine in thyroid cancer cells are mediated by mitochondrial pathway, and involved in the regulation of mutant p53 and up-regulation of p21 and Bcl-xL expression. More importantly, mutant p53 and Bcl-xL not only function as apoptosis regulators but also are critical proteins for iodine to influence thyroid cancer transformation. In the iodine-mediated apoptosis, the transient activation of MAPKs not only appears to be anti-apoptotic, but also functions in the regulation of cell differentiation, likely through modulating the levels of the mutated p53 or Bcl-xL. The data of this study provide some molecular evidence to explain why insufficient iodine intake is a risk factor for thyroid cancer, and generate some novel targets that may be potential for the treatment of this malignancy.

CHAPTER III: IODINE MODULATES DOXORUBICIN AND SODIUM BUTYRATE-INDUCED APOPTOSIS IN THYROID CANCER CELLS THROUGH ERK/P38-MEDIATED BCL-XL EXPRESSION

3.1 Introduction

Thyroid cancers which origins from follicular thyroid cells have three main subtypes: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC). The former two belong to differentiated thyroid cancer (DTC) with well prognosis, while, ATC is a rare but aggressive subtype of thyroid cancers with median survival of 4-12 months from the time of diagnosis (Are and Shaha, 2006; Braverman and Utiger, 2005). Multimodal therapies such as surgical resection, radiotherapy and chemotherapy have been used, but no satisfactory treatment has been established yet. Indeed, more than 50% of patients with ATC are metastatic and they frequently resist radiotherapy (Pasieka, 2003). Therefore, it needs to enhance the efficacy of chemotherapy for ATC. Doxorubicin (Dox) is known to be the most effective single cytotoxic drug against tumors by introducing double-strand breaks on DNA in proliferating cells to trigger apoptosis (Swift et al., 2006). However, the clinical impact of Dox on thyroid cancer remains limited due to a high degree of chemoresistance (Pasieka, 2003). Histone deacetylase (HDAC) inhibitors, such as sodium butyrate (NaB), have emerged as a promising new class of antitumor agents through the hyperacetylation of histiones and subsequent relaxation of chromatin, enhancing the cytotoxicity of drugs to target DNA in cancer cells including ATC (Catalano et al., 2006; Kim et al., 2003; Massart et al., 2005). Recently, there is a report showing that successful treatment of

ATC can be achieved by the combination of the HDAC inhibitor with Dox and other treatments, although the mechanism is unknown (Noguchi et al., 2009).

Iodine is one basic element of thyroid via modulating cell growth and development. Previous studies have shown that sufficient iodine supply could prevent the development of thyroid cancer (Farahati et al., 2004; Krohn et al., 2007; Maier et al., 2007). However, iodine metabolism is impaired in thyroid cancer, which reduces bioavailability of iodine and limits the effect of radioactive ^{131}I therapy to benefit the malignancy of thyroid (Schlumberger et al., 2007; Van Nostrand and Wartofsky, 2007). Recent studies indicated that chemotherapeutic agents, Dox and HDAC inhibitors, could enhance iodide uptake via restoring the function of sodium iodide symporter (NIS) gene, which majoring iodide transport (Haugen, 2004; Kim et al., 2007b). Thus, this study was based on the believing that iodine should be taken into account in thyroid cancer chemotherapy.

Members of the Bcl-2 family proteins are essential regulators of apoptotic process induced by various stimuli, including chemotherapeutic agents (Chipuk et al., 2008; Weintraub et al., 2004). Bcl-xL, an anti-apoptotic protein of Bcl-2 family, is highly expressed in a variety of thyroid cancers, contributing to chemoresistance in thyroid cancer treatment (Martinez-Brocca et al., 2008). Moreover, Bcl-xL up-regulated by mitogen-activated protein kinases (MAPKs) pathways have been conferred as one important pathway in chemoresistance (Wang et al., 2009), and HDAC inhibitors could effectively reverse the resistance to apoptosis through down-regulating Bcl-xL expression

(Hajji et al., 2008; Natoni et al., 2005). Previous studies also demonstrated that iodine could modulate Bcl-xL expression through activated MAPKs pathways. Thus, we hypothesize that the chemotherapeutic agents (Dox and HDAC inhibitors) and iodine function in concert to regulate Bcl-xL and MAPKs signaling pathways and trigger apoptosis in thyroid cancer cells. Therefore, the study will examine the effects of iodine and chemotherapeutic agents Dox and HDAC inhibitor NaB on apoptosis in ATC cells.

3.2 Materials and methods

3.2.1 Cell culture and treatment

Human ATC cell line ARO cells were cultured in RPMI 1640, supplemented with 1% L-glutamine, and 100 units/ml penicillin-100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37°C and 5% CO_2. Fresh medium was added to the cells the day before experiments. ARO cells were treated with I_2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h. For ARO cells treated with NaB plus other agents, the cells were pretreated with 4 mM NaB for 6 h before the addition of other agents (Catalano et al., 2009; Kim et al., 2005). All reagents used in the cell culture were purchased from Gibco (Grand Island, NY).

3.2.2 Reagents and antibodies

Primary antibodies against Bax, Bak, caspase 3, phospho-p44/42 MAPK(Erk1/2) (Thr202/Tyr204), p44/42 MAPK(Erk1/2) and phospho-p38 MAPK (Thr180/Tyr182) (28B10) were obtained from Cell Signaling Technology (Beverly, MA). The other primary antibodies against Cyto C, Bcl-xL (7B2.5), JNK (F-3), phospho-JNK (Thr183/Tyr185) (G-7), p38 (A-12), and β-Actin (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horse radish peroxidase (HRP) labeled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Iodine (I_2), NaB, and Dox were all purchased from Sigma (Sigma-Aldrich, MO). ERK inhibitor PD98059 and p38 inhibitor SB203580 were obtained from Promega (Madison, WI).

3.2.3 Measurement of mitochondrial membrane potential (Δψm) by flow cytometry

Forty nM DioC6 (Sigma-Aldrich, St. Louis, MO) were incubated with treated cells at indicated time-points for 15 min at 37°C. The harvested cells were washed with ice-cold PBS and analyzed by flow cytometry at an excitation wavelength 488 nm and an emission wavelength 525 nm by Becton Dickinson FACS Vantage machine. Cells with low $\Delta\psi_m$ were presented as the percentage of the total cell population. The CellQuest software (Verity Software House, Topsham, ME) was used to analyze the data.

3.2.4 Apoptosis analysis

Apoptosis assay was performed using APO-DIRECT™ TUNEL ASSAY kit (Chemicon international, San Diego, CA). At the indicated time points, cells were harvested and fixed in 1% paraformaldehyde for 60 min at 4°C at first, followed by washing with ice-cold PBS and fixing in 70% (v/v) ethanol overnight at -20°C. Then the cells were treated with various reagents for a designed period according to manufacture's instruction. Finally, the cells were analyzed by flow cytometry using a 488-nm argon laser for excitation by FACS Vantage machine (Becton Dickinson, Franklin Lakes, NJ). The CellQuest software (Verity Software House, Topsham, ME) was used to analyze the data.

3.2.5 Western blot analysis

Total protein was extracted by RIPA buffer [1×PBS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), pH 7.6 with protease inhibitors 0.1% aprotonin, 100 μM sodium orthovanadate and 100 μg/ml phenylmethy-sulphonyl-fluoride (PMSF)]. Cytosolic protein fraction was isolated according to a previous procedure (Li et al., 2006). Protein concentration was determined by Bio-Rad Dc reagent (Bio-Rad, CA). Ten μg of the cytosolic protein or 30 μg of the total protein was separated on 8%-15% SDS/polyacrylamide gels and then transferred onto a nitrocellulose membrane (Amersham Biosciences, NJ). The membrane was

blocked with 5% nonfat milk and then probed with specific primary antibody overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature after washing. The signals were visualized with ECL Kit (GE healthcare, UK). All Western blot analyses were performed at least twice independently.

3.2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from thyroid cancer cells using Trizol reagent (Invitrogen, CA) following the instructions provided by manufacturer. First-strand DNA synthesis was primed with oligo(dT) and completed using Reverse Transcription System (Promega, Madison, WI) followed by polymerase chain reaction (PCR). Primer sequences were as follows: Bcl-xL forward (367F), 5'-CAT CAC CCC AGG GAC AGC ATA TC-3'; reverse (367R), 5'-TGG TCA TTT CCG ACT GAA GAG TG-3'; G3PDH forward (435F), 5'-CAC TGC CAC CCA GAA GAC TG-3'; reverse (435R), 5'-TCC ACC ACC CTG TTG CTG TA-3'. The PCR conditions are as follows: an initial 3 min-denaturation at 94°C, followed by 30 cycles of amplification at 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. RT-PCR products were visualized by ethidium bromide staining on 1.5% agarose gels.

3.2.7 Transient transfections

ARO cells were plated in 6-well plates 24 h prior to transfection and grown in 70% confluence. Plasmid p3Bcl-xL, which contains full length of human Bcl-xL sequence

(NM_138578), was used in transient transfection (Miao et al., 2006). The empty vector pcDNA3.1(+) (Invitrogen, CA) was used as a control. For each well of cells to be transfected, 1 μg of plasmid was diluted with 100 μl of Opti-MEM I Reduced Serum Medium (Invitrogen, CA), and then 3 μl of Lipofectamine™ 2000 (Invitrogen, CA) was diluted with 100 μl of Opti-MEM I Reduced Serum Medium (Invitrogen, CA) as manufacturer recommendation. After 6 h incubation at 37°C, the medium was replaced with fresh growth medium containing serum and the cells were incubated for another 18 h before use.

3.2.8 Statistical analysis

Differences between groups were examined for statistical significance using Student's t-test. All p-values are two-sided and $P<0.05$ was considered statistically significant. All statistical calculations were performed by the SPSS software (version 13.0, IL). The data were presented as mean ± SD from at least three independent experiments.

3.3 Results

3.3.1 Iodine modulates apoptosis induced by Dox, NaB and NaB-Dox through mitochondrial pathway

To determine the effect of iodine and chemotherapeutic agents in combination on the cell death of thyroid cancer cells, TUNEL assay was used to assess apoptotic cells. ARO cells were treated with I_2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h. For ARO cells treated with NaB plus other agents, the cells were pretreated with 4 mM NaB for 6 h before the addition of other agents. As illustrated in Fig. 3.1A, apoptotic cells in Dox- and NaB-treated groups were moderate, 6.93 ± 2.08% and 30.27 ± 8.38% respectively. The combination of NaB and Dox (NaB-Dox) sharply enhanced the apoptotic cells to 80.81 ± 5.54%. The apoptosis rate of ARO cells treated by 50 μM of I_2 alone was mild (8.22 ± 1.97%), and it counteracted apoptosis induced by Dox (Dox: 6.93 ± 2.08%, *vs.* Dox-I_2: 2.88 ± 0.7%, $P<0.05$) and NaB plus Dox (NaB-Dox: 80.81 ± 5.54%, *vs.* NaB-Dox-I_2: 35.62 ± 4.38%, $P<0.01$), although NaB-induced apoptosis was promoted (NaB: 30.27 ± 8.38%, *vs.* NaB-I_2: 51.62 ± 1.36%, $P<0.05$).

To discover whether the mitochondrial death pathway was involved in the process, the reduction rate of mitochondrial membrane potential (MMP) was examined. The result was quite matched to the TUNEL data (Fig. 3.1B). Western blot analysis revealed that administration of NaB-Dox resulted in the strongest release of Cyto C into cytosolic

fraction and activation of caspase 3 and the effect were partly inhibited when NaB-Dox co-administrated with iodine, while, other treatments showed minimal effects (Fig. 3.1C). The Western blot data appeared to be concordant with the results obtained by TUNEL and MMP measurement.

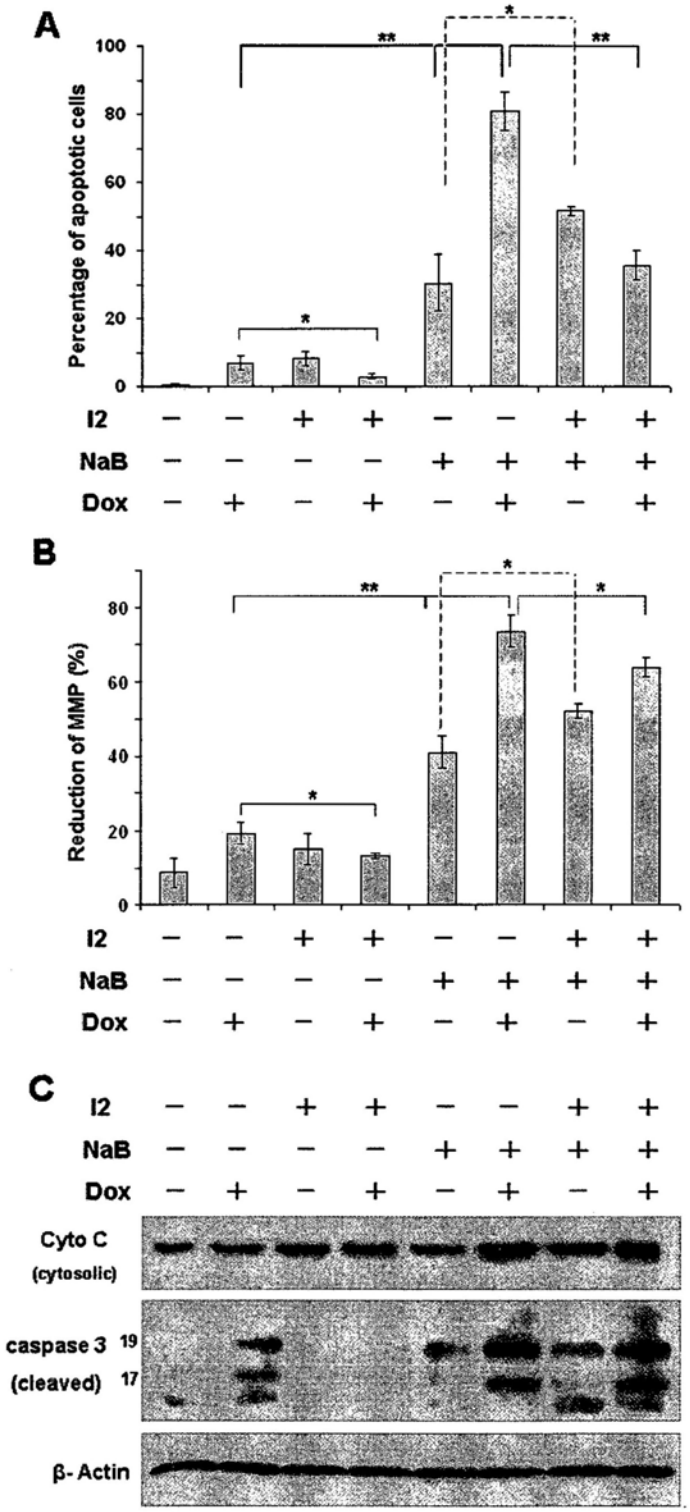

A
Percentage of apoptotic cells
100
80
60
40
20
0
**
*
*
**
I2 − − + + − − + +
NaB − − − − + + + +
Dox − + − + − + − +
B
Reduction of MMP (%)
80
60
40
20
0
**
*
*
*
I2 − − + + − − + +
NaB − − − − + + + +
Dox − + − + − + − +
C
I2 − − + + − − + +
NaB − − − − + + + +
Dox − + − + − + − +
Cyto C
(cytosolic)
caspase 3 19
(cleaved) 17
β- Actin

Fig. 3.1 Iodine modulates apoptosis induced by Dox, NaB and NaB-Dox through the mitochondrial pathway. ARO cells were treated with I_2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h. For ARO cells treated with NaB plus other agents, the cells were pretreated with 4 mM NaB for 6 h before the addition of other agents. **A.** The apoptotic cells were assessed by flow cytometry with TUNEL assay. The data represent mean ± SD of three independent experiments, $*P<0.05$, $**P<0.01$. **B.** The percentage of cells exhibiting reduction of mitochondrial membrane potential (MMP) was assayed by flow cytometry after staining the cells with DioC6. The data represent mean ± SD of three independent experiments, $*P<0.05$, $**P<0.01$. **C.** Total protein and cytosolic fractions were isolated after treatments and Western blot was used to detect Cyto C and cleaved caspase 3 expression. Experiments were repeated at least twice.

3.3.2 The expression of Bcl-xL in ARO cells treated by iodine, Dox and NaB

To explore whether pro-apoptotic proteins Bax/Bak and anti-apoptotic protein Bcl-xL are involved in apoptosis induced by Dox, NaB and NaB-Dox, with or without iodine, Bax/Bak and Bcl-xL expressions were detected by Western blot. As shown in Fig. 3.2A, the level of Bax in each group was not significantly altered. Bak was up-regulated in all groups treated with NaB, but there was no significant difference between Dox and Dox-I_2, NaB and NaB-I_2, or NaB-Dox and NaB-Dox-I_2. Compared with the control, Bcl-xL was down-regulated by various treatments. Moreover, Bcl-xL expression in Dox-I_2 was higher than Dox, and NaB-Dox-I_2 was higher than NaB-Dox, while NaB-I_2 was lower than NaB. Similar results were obtained by RT-PCR (Fig. 3.2B). The expression pattern of anti-apoptotic protein Bcl-xL was matched with the changes in apoptotic cells detected by TUNEL, the reduction of MMP, the release of Cyto C and the activation of caspase 3, suggesting that the reduction of Bcl-xL might be a main mechanism contributed to apoptosis induced by iodine, Dox, and NaB, alone or in combination.

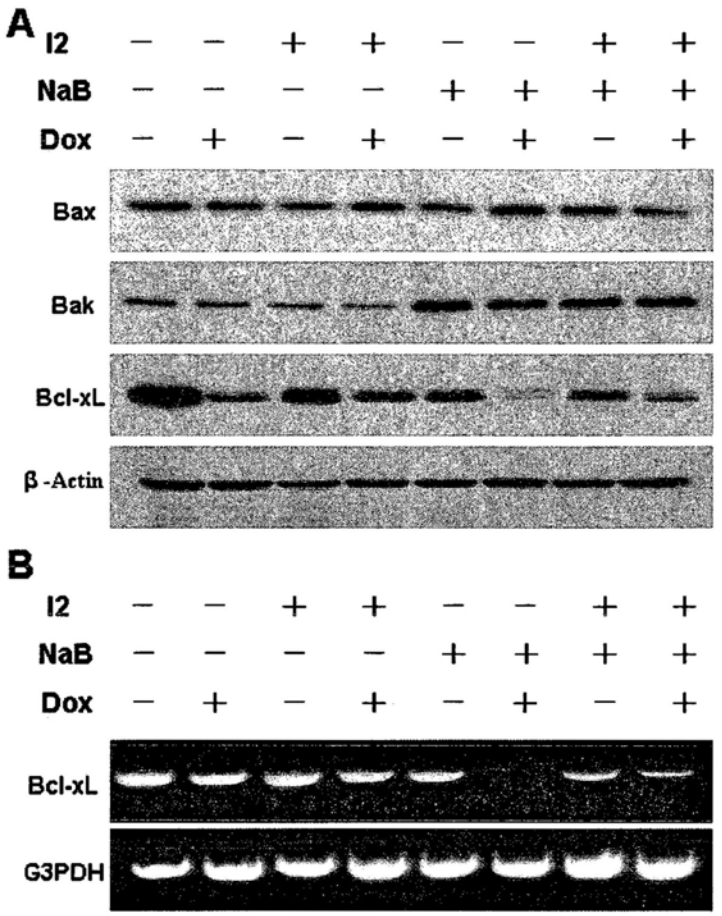


Fig. 3.2 Expression of Bcl-xL in ARO cells treated with iodine, Dox and NaB. ARO cells were treated with I2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h. For ARO cells treated with NaB plus other agents, the cells were pretreated with 4 mM NaB for 6 h before the addition of other agents. **A.** Total protein was isolated after treatment and Western blot was used to detect Bax, Bak and Bcl-xL expression. Experiments were repeated at least twice. **B.** Total RNA was prepared from ARO after treatment and RT-PCR was used to detect Bcl-xL expression. G3PDH was used as a control and experiments were repeated at least twice.

3.3.3 Bcl-xL overexpression blocks apoptotic effects

To verify the role Bcl-xL in the apoptosis induced by iodine and the chemotherapeutic agents, Bcl-xL protein in ARO cells was overexpressed (Fig. 3.3A). Then MMP reduction and apoptotic cells were assayed by flow cytometry. As shown in Fig. 3.3B and 3.3C, MMP reduction and apoptotic cells in ARO transfected with Bcl-xL were decreased in each group in varying degrees. Western blot analysis was used to assess the release of Cyto C and activation of caspase 3 (Fig. 3.3D). ARO cells transfected with empty vector and then treated by NaB-Dox were used as control. Comparing with the control, the levels of both Cyto C and caspase 3 were decreased in ARO cells transfected with Bcl-xL, suggesting that Bcl-xL overexpression could inhibit apoptosis.

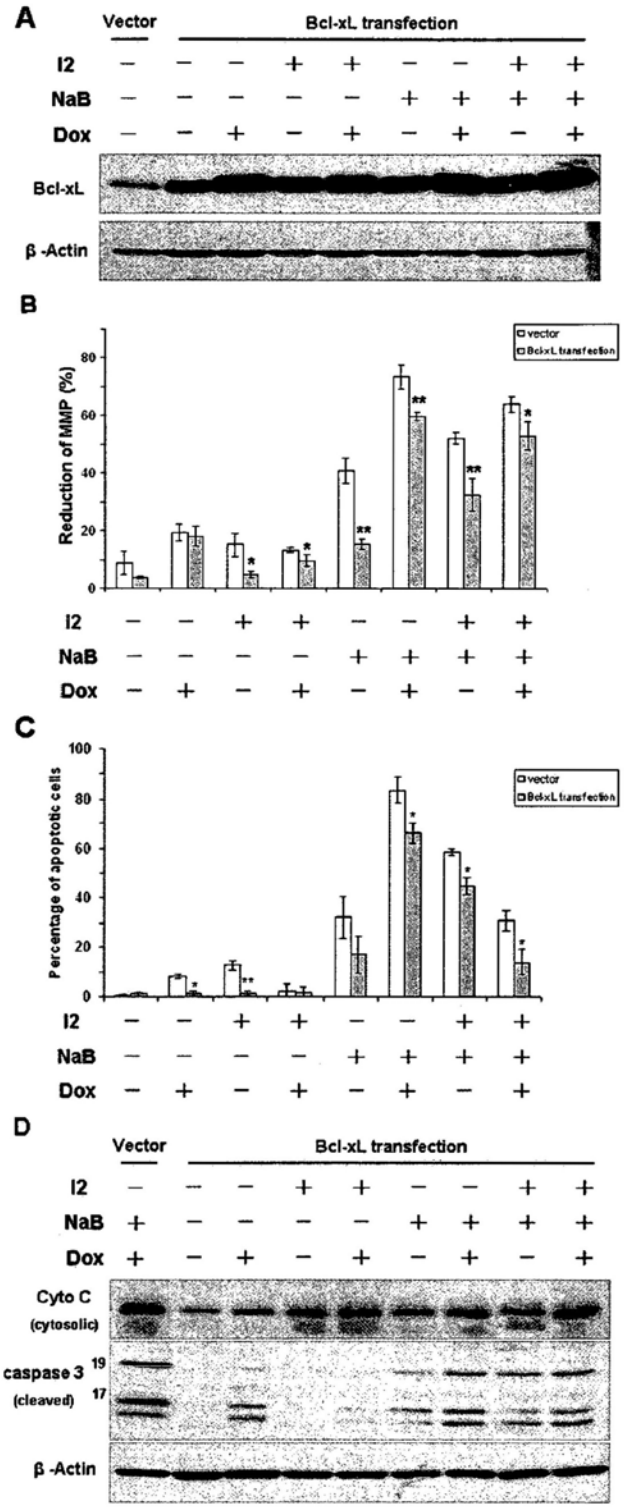

A
Vector
Bcl-xL transfection
I2
NaB
Dox
Bcl-xL
β -Actin
B
vector
Bcl-xL transfection
Reduction of MMP (%)
I2
NaB
Dox
C
vector
Bcl-xL transfection
Percentage of apoptotic cells
I2
NaB
Dox
D
Vector
Bcl-xL transfection
I2
NaB
Dox
Cyto C
(cytosolic)
caspase 3
(cleaved)
19
17
β -Actin

Fig. 3.3 Bcl-xL overexpression blocks apoptosis. ARO cells were transiently transfected with Bcl-xL or empty vector plasmid. The transfected ARO cells were treated with I_2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h. For ARO cells treated with NaB plus other agents, the cells were pretreated with 4 mM NaB for 6 h before the addition of other agents. **A.** Total protein was isolated after transfection and treatments, and then Western blot was used to detect Bcl-xL expression. ARO cells transfected with vector were used as control. Experiments were repeated at least twice. **B.** After transfection and treatment, MMP reduction of ARO cells was assayed by flow cytometry after staining the cells with DioC6. ARO cells transfected with empty vector were used as control. The data represent mean ± SD of three independent experiments, $*P<0.05$, $**P<0.01$. **C.** After transfection and treatments, TUNEL assay was used to assess the apoptotic cells. ARO cells transfected with vector were used as control. The data represent mean ± SD of three independent experiments, $*P<0.05$, $**P<0.01$. **D.** Total protein and cytosolic protein were isolated after transfection and treatments, and then Western blot was used to detect caspase 3 and Cyto C expression. ARO cells transfected with empty vector and treated by NaB-Dox were used as control. Experiments were repeated at least twice.

3.3.4 ERK and p38, but not JNK, are implicated in apoptosis induced by iodine, Dox and NaB

To explore whether MAPKs signaling pathways are involved in the process, an initial examination was carried out on the expressions of ERK1/2, p38 and JNK1/2 expression in ARO cells by Western blot. As shown in Fig. 3.4, it could be observed that the levels of both phospho-ERK (p-ERK) and phospho-p38 (p-p38) were modulated by various treatments; however the expression of phospho-JNK (p-JNK) was not significantly affected. ERK was activated in cells treated with Dox, NaB, and NaB-I_2, but inhibited in those treated with I_2, NaB-Dox, and NaB-Dox-I_2. In contrast, levels of p-ERK varied with treatments. It was reduced markedly in cells treated with NaB-Dox, compared with cells treated with Dox or NaB alone. Moreover, p-ERK level in Dox-treated ARO cells was higher than in Dox-I_2-treated ARO cells; On the contrary, p-ERK level in NaB-Dox-treated ARO cells was lower than in NaB-Dox-I_2-treated ARO cells, although the level of p-ERK did not differ significantly between cells treated with NaB and those treated with NaB-I_2. Compared with control, the level of p-p38 was increased in cells treated with NaB-Dox, but decreased in all other groups. Notably, p-p38 levels in Dox and NaB-Dox treated ARO cells were inhibited when combined with iodine.

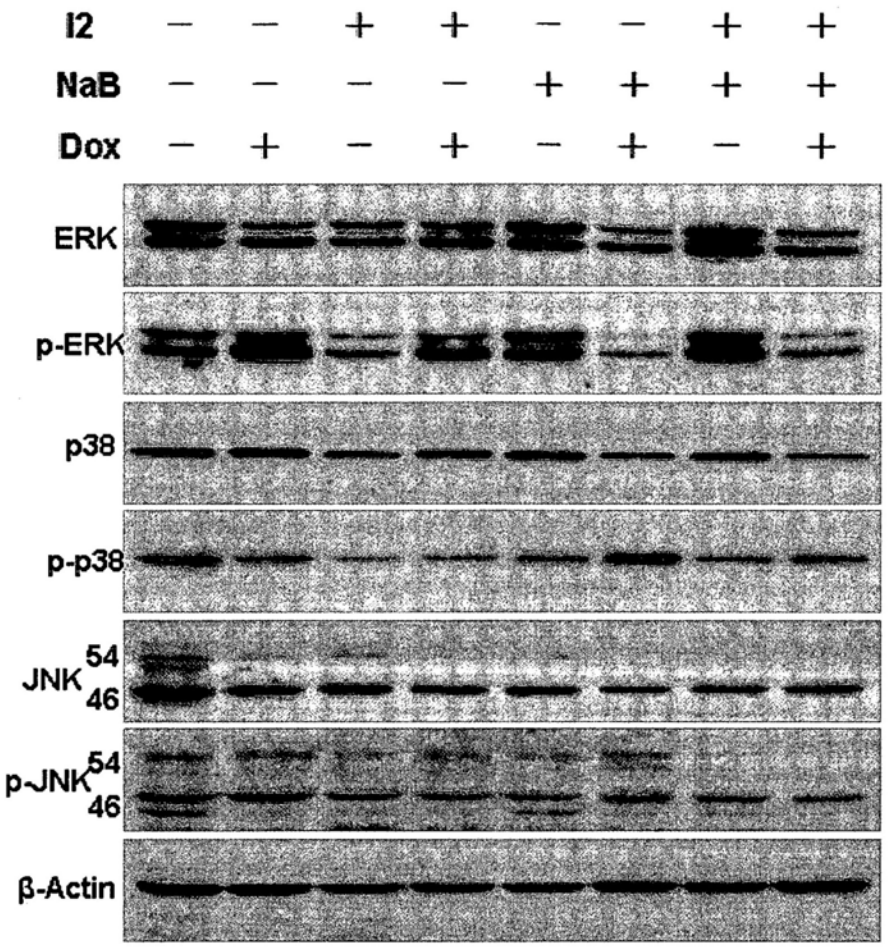


Fig. 3.4 ERK and p38, but not JNK, are implicated in apoptosis induced by iodine, Dox, and NaB. ARO cells were treated with I_2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h. For ARO cells treated with NaB plus other agents, the cells were pretreated with 4 mM NaB for 6 h before the addition of other agents. Total protein was isolated after treatment and Western blot was used to detect ERK, phospho-ERK (p-ERK), p38, phospho-p38 (p-p38), JNK and phospho-JNK (p-JNK) expression. Experiments were repeated at least twice.

3.3.5 ERK and p38 pathways modulate Bcl-xL expression in iodine, Dox and NaB treated ARO cells

To determine whether ERK and p38 were involved in the expression of Bcl-xL in ARO cells, the specific inhibitors of ERK and p38 were used. Before ARO cells were treated with I_2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h, ARO cells were left untreated (Fig. 3.5A), or pretreated with ERK specific inhibitor PD98059 (40 μM) (Fig. 3.5B) and p38 specific inhibitor SB203580 (40 μM) (Fig. 3.5C) for 1 h to block the corresponding pathways. Western blot analysis was performed to assess the expression of Bcl-xL after ERK or p38 were respectively blocked. As shown in Fig. 3.5B and 3.5C, there was no change in Bcl-xL levels in ARO cells treated by either PD98059 or SB203580 alone. However, in the presence of ERK inhibitor PD98059, the level of Bcl-xL was decreased, especially in cells treated by Dox alone. When p38 pathway was inhibited by SB203580, the level of Bcl-xL was decreased in cells treated by Dox alone but significantly up-regulated in cells treated with NaB-Dox (Fig. 3.5C).

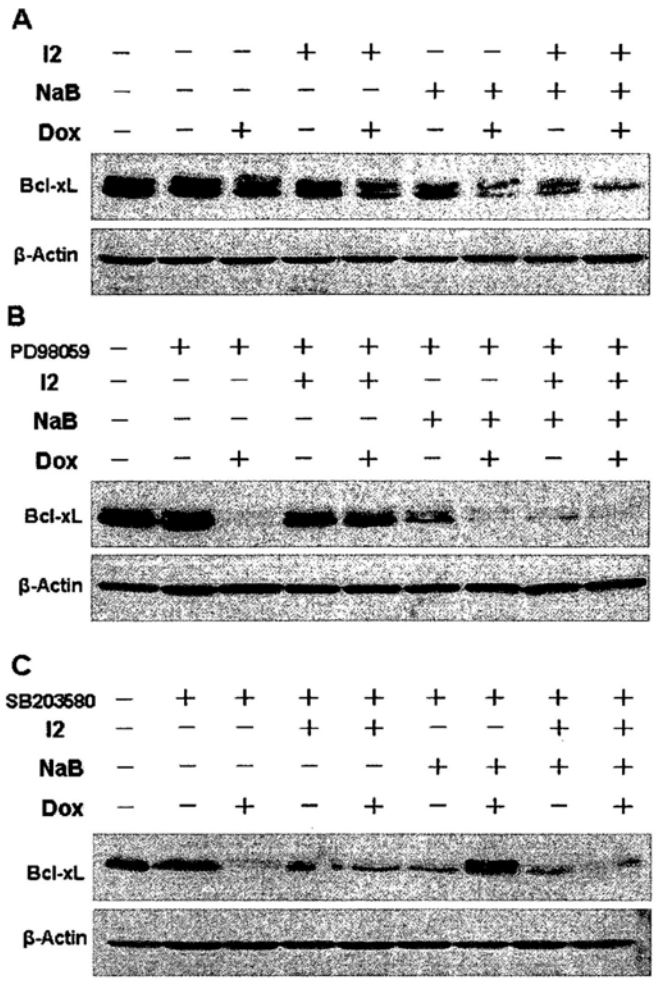


Fig. 3.5 ERK and p38 modulate Bcl-xL expression in ARO cells treated with iodine, Dox, and NaB. Before ARO cells were treated with I_2 (50 μM), NaB (4 mM) and Dox (1 μg/ml), alone or in combination for 48 h, ARO cells were left untreated (**A**), or pretreated with ERK specific inhibitor PD98059 (40 μM) (**B**) and p38 specific inhibitor SB203580 (40 μM) (**C**) for 1 h to block the corresponding pathways. Total protein was isolated after treatments and Western blot was used to detect Bcl-xL expression. Experiments were repeated at least twice.

3.4 Discussion

ATC is very aggressive and the ability of tumor cells to escape the cytotoxic activity of chemotherapeutic agents may result from molecular alterations in apoptosis, leading to chemoresistance (Are and Shaha, 2006). Dox is a valid chemotherapeutic agent for thyroid cancer patients, especially for patients with advanced or metastatic carcinomas. However, the clinical impact is always limited due to a high degree of chemoresistance (Pasieka, 2003). Recently, HDAC inhibitors have emerged as a promising new class of antitumor agent, due to the function that sensitizes the cytotoxicity of Dox through hyperacetylation of histones and subsequent relaxation of chromatin (Marks et al., 2004; Massart et al., 2005). The present findings indicate that co-administration of NaB and Dox enforces the lethal effect of either Dox or NaB, which is consistent with the earlier studies that the cytotoxicity of HDAC inhibitors and Dox in combination can be enhanced for treatment of ATC (Catalano et al., 2006; Noguchi et al., 2009). In fact, thyroid cancer is unique among cancers due to thyroid cells are the only cells that have the ability to absorb iodine. Subsequent studies have indicated that iodine could induce apoptosis *in vitro* and prevent tumor growth *in vivo* (Garcia-Solis et al., 2005; Shrivastava et al., 2006). However, how iodine affects the efficiency of chemotherapeutic agents is unknown. The findings of this study indicate that iodine counteracts Dox- and NaB-Dox- but promotes NaB-induced apoptosis through the mitochondrial pathway. The findings provide evidence that the influence of iodine on the apoptotic effect of chemotherapeutic agents is different although they are all modulated via the mitochondrial pathway. To my best knowledge, this is the first report to put forward that iodine should be taken into account in thyroid cancer chemotherapy.

Bcl-xL is a well-known anti-apoptotic regulator, which protects cells from apoptosis induced by chemotherapeutic agents. The increase of Bcl-xL may result in chemoresistance in cancer therapy (Chipuk et al., 2008; Weintraub et al., 2004). Bcl-xL is frequently expressed in thyroid cancer, suggesting that it may have a role in thyroid cancer chemotherapy (Martinez-Brocca et al., 2008). This study revealed that Bcl-xL was down-regulated at the protein and the transcriptional levels by various treatments. Among these treatments, NaB-Dox was the most effective, followed by NaB and Dox. The finding suggests that these treatments induce apoptosis of thyroid cancer cells probably via the reduction of Bcl-xL. The result is in line with reports showing that the predominant knockdown of Bcl-xL is an effective pathway to overcome chemoresistance in cancer therapy and it may potentiate the activity of cytotoxic drugs (Bai et al., 2005; Shoemaker et al., 2006). The role of Bcl-xL as an anti-apoptotic molecule in thyroid cancer chemotherapy is further elucidated by the overexpression of Bcl-xL since its overexpression significantly diminishes apoptosis induced by NaB-Dox.

Moreover, this research also found that Bcl-xL expression was decreased by moderate dose of iodine. Earlier studies have evidenced that Bcl-xL expression is higher in DTC than in ATC (Martinez-Brocca et al., 2008; Stassi et al., 2003). And the incidence of DTC in areas with sufficient iodine supply is more popular than those with deficient iodine supply (Dijkstra et al., 2007; Farahati et al., 2004). These results may indicate that Bcl-xL is the key molecule for iodine to influence the development of thyroid cancer. Besides, this study also found that the level of Bcl-xL was further inhibited when the cells were co-treated with iodine and NaB, compared with cells treated by NaB alone. However, co-treatment of iodine and Dox significantly reversed the Dox-induced

down-regulation of Bcl-xL. These findings suggest that iodine may differentially affect Bcl-xL expression in thyroid cancer cells treated with chemotherapeutic agents. Taken together, it appears that Bcl-xL is a main molecule involved in not only iodine-related thyroid cancer development, but also thyroid cancer chemotherapy.

The three main subfamily members of MAPKs, ERK1/2, p38, and JNK1/2, have been reported to play important roles in mediating proliferation and apoptosis of tumor cells including thyroid cancer cells (Liu et al., 2008; Wada and Penninger, 2004). In this study, results demonstrated that ERK and p38, but not JNK, were involved in apoptosis of ATC cells treated with Dox, NaB and iodine, alone or in combination. Various studies have shown that both ERK and p38 can be either anti- or pro-apoptotic through modulating apoptosis regulators such as Bcl-xL (Bachelor and Bowden, 2004; Wang et al., 2009). Remarkably in this study, it was observed that Dox down-regulated Bcl-xL expression and that this down-regulation of Bcl-xL was maximized in the presence of either ERK or p38 inhibitor, suggesting that the inhibition of both ERK and p38 pathways play a positive role in the down-regulation of Bcl-xL. In the separated experiment, it was found that Dox itself inhibited p38 but activated ERK. Since the final consequence of Dox on Bcl-xL expression is its down-regulation, the Bcl-xL expression appears to be regulated dominantly by p38 rather than ERK. Therefore, these findings could be used to explain why Dox treatment resulted in the reduction of p38, the enhancement of ERK, but the decrease of Bcl-xL. These findings also suggest that Bcl-xL expression is modulated by a fine balance between ERK and p38 pathway, which was also evidenced in the cells exposed to NaB-Dox. Compared with cells treated by Dox, ERK was inhibited and p38 was activated in the cells treated with NaB-Dox. The

inhibition of ERK and the activation of p38 shift the balance between ERK and p38 in favor of the latter, which leads to a significant decrease in the expression of Bcl-xL and a marked increase in apoptosis. In the presence of iodine, the pro-apoptotic effect of NaB-Dox was inhibited but the percentage of apoptotic cells was still higher than the control. Taken together, the balance between ERK and p38 was considered as the key point in controlling cell survival and death through modulating Bcl-xL expression in cell model tested. The high ERK/p38 ratio favors Bcl-xL up-regulation and chemoresistance, whereas the low ERK/p38 ratio benefits Bcl-xL down-regulation and chemosensitivity (Fig. 3.6) (Aguirre-Ghiso et al., 2003; Choi et al., 2007). Iodine status has been considered as one factor in thyroid cancer incidence: iodine sufficiency benefits DTC while iodine deficiency favors ATC (Dijkstra et al., 2007; Farahati et al., 2004), but the mechanism is unclear. This study shows that ERK and p38 were inhibited by iodine, suggesting ERK and p38 may contribute to iodine–mediated thyroid cancer transformation. Thus, iodine not only participates in chemotherapy, but also contributes to thyroid cancer transformation.

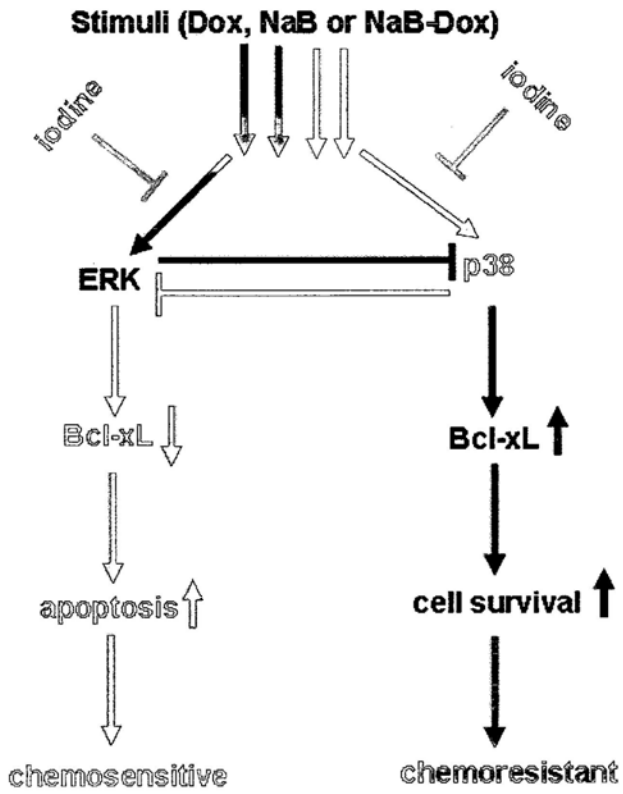


Fig. 3.6 The balance between ERK and p38 determines cell survival and death in ARO cells through modulating Bcl-xL expression. In response to stimuli, the levels of ERK and p38 will be altered accordingly. When ERK activity is dominant over p38 activity, Bcl-xL expression is up-regulated, the tumor cells tend to survive and become chemoresistant (blue). However, when p38 activity is dominant over ERK activity, Bcl-xL expression is down-regulated, the tumor cells are prone to apoptosis and become chemosensitivity (orange). Meanwhile, moderate doses of iodine may inhibit activity of ERK as well as p38 and thus plays a role in the fine-regulation of apoptosis in thyroid cancer cells treated with Dox, NaB or both in combination (purple).

It is well known that the effect of iodide depends on iodide-specific transportation genes in thyroid such as NIS (Zhang et al., 2003). However, the function or expression of iodide-specific transportation genes such as NIS is usually impaired in thyroid cancer cells (Dohan et al., 2003; Schlumberger et al., 2007). Therefore, one may wonder how iodide or iodine enters the cells used in the present experiment to exert its effects. It has been reported that excess iodine generation and oxidation of ionic iodine by endogenous peroxidases are key steps in iodide-induced apoptosis (Smyth, 2003; Vitale et al., 2000), and that iodine intake can be independent of the iodide-specific transportation genes such as NIS (Garcia-Solis et al., 2005; Shrivastava et al., 2006).

Taken together, this research gives demonstration that iodine plays critical roles in apoptosis of thyroid cancer cells, and identifies the underlying mechanism of how NaB sensitizes Dox cytotoxicity in ARO cells. The balance between ERK and p38 appears to have a critical role in determining cell survival and death, likely through the modulation of Bcl-xL expression in thyroid cancer cells. This study provides some new insights into the apoptotic effect of iodine on thyroid cancer cells. Thus, iodine status should be taken into account in thyroid cancer chemotherapy.

CHAPTER IV: SUMMARY AND FUTURE PROSPECTS

4.1 Summary

This research studies how iodine influences apoptosis of thyroid cancer *in vitro* through apoptotic pathway. Two key results of this study are discovered as follows.

(1) Iodine induces apoptosis through the regulation of MAPKs-related p53, p21, and Bcl-xL in thyroid cancer cells.

Iodine has long been associated with thyroid tumorigenesis and therapy. However, the effects induced by iodine and the molecular mechanisms involved remain scarcely studied in thyroid cancer, which provides the valuable opportunity for this research. This research investigated the apoptotic effects of iodine in thyroid cancer by studying apoptosis. The results showed that apoptosis induced by iodine was mitochondrial-mediated, with the loss of mitochondrial membrane potential, Bak up-regulation, caspase 3 activation and cytochrome C release from mitochondria. Iodine treatment decreased the level of mutant p53 including the R273H mutant that possesses anti-apoptotic features, but increased the p21 level. The block of p21 significantly prevented iodine-induced apoptosis. Iodine also stimulated the activation of the subfamily members of MAPKs (ERK1/2, p38 and JNK1/2). The results showed the three subfamily members of MAPKs all worked as anti-apoptotic factors. Surprisingly, iodine promoted instead of suppressed the expression of anti-apoptotic protein Bcl-xL. The increase of Bcl-xL was likely to compensate the damage induced by iodine since the inhibition of Bcl-xL accelerated iodine-mediated apoptosis. Collectively, research data demonstrated that iodine induced mitochondrial-mediated apoptosis in thyroid cancer cells. This apoptotic pathway was involved in the activation of MAPKs pathways, which may subsequently up-regulate p21, Bak, and

down-regulate anti-apoptotic mutant p53. The findings provide solid molecular evidence to explain the epidemiological observation that iodine insufficiency promotes the thyroid tumor development. It may also reveal some novel molecular targets for the treatment of thyroid cancer.

(2) Iodine modulates doxorubicin and sodium butyrate-induced apoptosis in thyroid cancer cells through ERK/p38-mediated Bcl-xL expression.

Anaplastic thyroid cancer (ATC) is lethal because of its rapid progression and poor response to chemotherapy and radioiodine therapy. Iodine is involved in the maintenance of thyroid function and the development of thyroid cancer, which provides the second opportunity for this research. This study examined the effect of iodine on the apoptosis of ATC cell line ARO treated with doxorubicin (Dox) and histone deacetylase inhibitor sodium butyrate (NaB). The cytotoxic effect of either Dox or NaB alone was limited, but co-administration of NaB and Dox (NaB-Dox) significantly increased mitochondrial-mediated apoptosis. The effects of iodine to apoptosis-induced by the two agents were diversified. Iodine reduced the apoptosis induced by Dox or NaB-Dox but promoted apoptosis induced by NaB. Further experiments showed that iodine exaggerated NaB-mediated Bcl-xL down-regulation. In contrast, it reduced the effect of Dox on the decrease of Bcl-xL. Meanwhile, iodine regulated the level of Bcl-xL in ERK- or/and p38-related pathways. The balance between ERK and p38 may determine the iodine-mediated Bcl-xL expression. The high ERK/p38 activity ratio up-regulated Bcl-xL expression and enabled the tumor cells to resist chemotherapy, whereas the low ERK/p38 down-regulated Bcl-xL and sensitized the tumor cells to chemotherapy. Taken together, iodine plays a critical role in apoptosis of thyroid cancer cells induced by chemotherapy. The balance between

ERK and p38 may determine cell survival and death through modulating Bcl-xL expression in thyroid cancer cells. The findings provide some new insights into the role of iodine in chemotherapeutic agent-induced apoptosis of thyroid cancer cells.

4.2 Future prospects

This research has evidenced that iodine can induce thyroid cancer apoptosis through MAPKs pathways-related Bcl-xL, p53 and p21 expression. Moreover, iodine can influence chemotherapeutic agents-induced apoptosis through ERK/p38-mediated Bcl-xL expression.

In addition to MAPKs pathways that involved in iodine-induced apoptosis, two other pathways, NF-κB and PI3K, correlated closely with apoptosis or cell proliferation, have been found related with iodine or iodide metabolism. NF-κB is one of the key factors controlling anti-apoptotic responses by transcriptional activation (Richmond, 2002). The molecular genetic events such as BRAF and PPARG can induce NF-κB activation, which makes it more important in thyroid cancer (Kato et al., 2006; Palona et al., 2006). In addition, iodide may attach itself in cell proliferation through NF-κB regulatory elements on multiple genes associated with growth (Taniguchi et al., 1998). Notably, *in vitro* and *in vivo* studies have indicated that NF-κB inhibitor is an effective pathway to induce thyroid cancer apoptosis (Starenki et al., 2004). PI3K signaling pathway is another important pathway in thyroid cancer because it can be activated by several genetic alterations such as BRAF, RAS and CTNNB1 (Abbosh and Nephew, 2005; Hou et al., 2007). In addition, studies have shown that PI3K inhibition could increase NIS functional expression, which is the key protein response iodide transport (Kogai et al., 2008). However, the underlying

mechanisms of how iodine works on NF-κB or PI3K signaling pathway to modulate apoptosis or cell proliferation in thyroid cancer are still unknown. Thus, the work to explore this unknown area will be valuable in many research fields. For instance, they will provide much more evidences for iodine on thyroid cancer development through apoptotic pathway, and also provide some new target pathways for thyroid cancer therapy.

Besides the correlations with signaling pathways involved in apoptosis and cell proliferation, iodine itself is also a critical factor in regulating the accumulation of I^- by the thyroid gland. Experiments show that moderate doses of iodine can inhibit the expression of NIS mRNA *in vivo* (Uyttersprot et al., 1997) an *in vitro* (Eng et al., 2001). It is well known that the expression and function of thyroid specific genes, NIS, TPO, TG, TSHR, and so on, are significant thyrocytes differentiation markers (Durante et al., 2007). Notably, these markers not only correlate with signaling pathways modulating apoptosis but also can be regulated by chemotherapeutic agents, such as Dox and HDAC inhibitors (Haugen, 2004; Kim et al., 2007b). However, the correlations among chemotherapeutic agents, signaling pathways and these thyroid specific genes are unknown. The work to find the possible relations and underlying mechanisms among them will benefit thyroid cancer chemotherapy and radioactive iodide therapy directly.

Genetic alterations in thyroid cancer are common, which are initiations for aberrant activation of cell signaling pathways and impaired balance between pro- and anti-apoptotic proteins. At present, genetic-targeted therapy of thyroid cancer has been constructed elementarily although the mechanism is unclear (Xing, 2009). BRAF mutation and RET rearrangement are popular in PTC (Hamatani et al., 2008;

Xing, 2005), PPARG rearrangement is common in FTC (Castro et al., 2006), while TP53 and CTNNB1 muation are prevalent in ATC (Farid, 2001; Garcia-Rostan et al., 2001). Epidemiological data have evidenced that sufficient iodine supply could promote PTC incidence and prevent the transformation from DTC to ATC (Wiseman et al., 2007; Wiseman et al., 2003). Therefore, the essential element of iodine in thyroid may work as a thyroid cancer risk factor via oncogenic proteins. Nevertheless, scare studies have disclosed the underlying mechanism. Thus, the work in this area may provide direct and solid evidence for iodine on thyroid cancer development.

However, the findings of this study are all obtained from *in vitro* experimental models, which need to be further clarified *in vivo*. Therefore, it is important to study how iodine works on thyroid cancer tumorigenesis and chemotherapy *in vivo*. The *in vivo* studies will also benefit from the antisense oligonucleotide of Bcl-2 and small-molecule antagonists of Bcl-2, such as ABT-737, which has been evidenced as one potential tumor growth inhibitor (Lessene et al., 2008; Waters et al., 2000). More importantly, the *in vivo* studies will provide direct evidence for thyroid cancer therapy.

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