The Role of Nuclear Factor-kappaB in the Laryngeal Cancer Cell Death Induced by Pteris Semipinnata L Extract

LO, Chun Shan

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Philosophy
in
Surgery

September 2008

Thesis/Assessment Committee

Professor POON Wai Sang (Chair)
Professor Alexander Chris VLANTIS (Thesis Supervisor)
Professor CHEN Gong George (Thesis Supervisor)
Professor Charles Andrew van Hasselt (Committee Member)
Professor GUO Zhu Ming (External Examiner)

Abstract

Chinese herbal medicine has been shown to be a potential anti-cancer drug by inducing differentiation, apoptosis, inhibiting angiogenesis, reversing multi-drug resistance through different mechanisms. Pteris semipinnata L (PsL), a Chinese herb, was traditionally used to treat enteritis, hepatitis and snake bites. The natural chemical compound ent-11-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F), an ethanolic extract of PsL, has been shown to carry anti-tumor activity by inducing apoptosis in human colon cancer, gastric cancer and thyroid cancer.

In cancer chemoprevention and chemotherapy, induction of apoptosis is an attractive strategy by introducing the anti-cancer drug either orally or injection. Since 5F can induce apoptosis as shown by many evidences, it can be employed as a potential apoptosis induction drug in laryngeal cancer therapy. Moreover, NF-κB, one of 5F target in human colon cancer cells, is one of the most important apoptosis regulators. Because NF-κB activity was increased in laryngeal cancer, its role in 5F-induced apoptosis might be pivotal. On the other hand, there are growing evidences showing that HPV was associated with laryngeal carcinogenesis. How 5F functions in HPV positive or negative laryngeal cancer cells is unknown.

In this project, three laryngeal cancer cell lines, UMSCC11A, UMSCC12 and HEp-2, were employed. HEp-2 is HPV18 positive while the others are HPV 18 negative. Our

results demonstrated that 5F inhibited laryngeal cancer cell growth in a does-dependent manner. It was noticed that HEp-2 was more resistant to 5F. E6 and E7 (encoded by HPV18) were reported to promote cell growth; therefore, it is conceivable that the presence of HPV18 may account for the resistance to the anti-proliferation of 5F of the HEp-2. We found that 5F suppressed the expression of E7 while the expression of E6 was not altered. Cleavage of pro-caspase 3 and poly (ADP-ribose) polymerase (PARP) and Annexin V assay confirmed that 5F induced apoptosis in laryngeal cancer cells. Moreover, 5F suppressed the NF-κB activity, via blockage of NF-κB nuclear translocation, leading to inhibition of its target gene products responsible for cell proliferation and apoptosis. With the results shown by NF-κB specific inhibitor, Bay (11-7082), we verified that NF-κB is responsible for laryngeal cancer cell survival.

In conclusion, we demonstrated that suppression of NF-κB activity involved in 5F-induced apoptosis in laryngeal cancer cells. This implicated that 5F may represent as a new potent agent, or in combination with other inhibitors, for treatment of laryngeal cancers by targeting NF-κB signaling pathway.

摘要

以往的研究已經顯示，中草藥可通過不同機制導致細胞分化、凋亡、抑制血管生成及逆轉多抗藥性，從而達到抗腫瘤作用。半邊旗是一種中草藥，一般用於治療腸炎、肝炎和毒蛇咬傷。研究證實，半邊旗的乙醇提取物，對映-11α-羥基-15 –氧代-貝殼杉-16-烯-19-酸(5F)，可以誘導人類結腸癌、胃癌和甲狀腺癌細胞凋亡。

在防癌和化療中，不論是口服或是注射抗癌藥物，誘導腫瘤細胞凋亡是一種有效的手段。而各種證據顯示，5F可以誘導腫瘤細胞凋亡，可能對喉癌的治療也有一定的作用。NF-κB是重要的凋亡調節因子之一，也是5F在結腸癌細胞的結合位點。由於NF-κB的活性在大部份腫瘤和喉癌細胞中有所增加，所以NF-κB在5F誘導的細胞凋亡中可能起到至關重要的作用。另外，不斷有研究發現，人類乳頭瘤病毒(HPV)與喉癌的發生有關。但是，5F在HPV陽性和因性的喉癌細胞中的作用尚未明確。

在目前的研究中，我們使用三種喉癌細胞系，包括UMSCC11A、UMSCC12和HEp-2。後者是HPV 18陽性細胞，而前兩者都是HPV 18陰性。我們的研究證實，5F可以抑制喉癌細胞生長，且呈明顯的劑量依賴性。值得注意的是，HEp-2對5F的耐藥性較強。有研究發現，由HPV 18編碼的E6和E7可促進細胞生長。因此，HPV18的存在可能是造成HEp-2對5F抗增殖作用耐受的原因。我們發現，5F抑制E7的表達，而E6的表達並不改變。caspase-3前體和多聚ADP核糖多聚酶(PARP) 裂解片段

的表達，以及Annexin V的結果也證實了5F可以誘導喉癌細胞凋亡。此外，5F可以通過阻斷NF-κB核轉移來抑制其活性，從而抑制其細胞增殖和凋亡的目標基因產物。通過添加NF-κB 特異性抑制劑—Bay (11-7082)的研究結果證實，NF-κB確實與喉癌細胞生存率有關。

由目前的研究可以得知，抑制NF-κB 的活性可能與5F誘導的喉癌細胞凋亡有關。結果表示，5F可能可以透過NF-κB 通路來治療喉癌。

Acknowledgements

I am greatly indebted to my supervisors Professor AC Vlantis and Professor George G. Chen for their continuous help and support during the course of this research and for their expert and careful reading of this thesis.

I would like to thank my colleagues for their assistance, encouragement and discussions. Special thanks are due to Ms. Ursula P.F. Chan, Mr. Rocky Ho, Ms. S.Y. Chun, Mr. Billy Leung, Dr. K.C. Leung, Miss Angela M.Y. Hui, Miss Angela B.Z. Liu, Miss Lydia K.W. Lung, Ms X.H. Liu, for their assistance and advice on my study. I would also like to thank all the other colleagues in Cancer Center, especially Jason and his colleagues in 302, who made my time in the Cancer Center enjoyable. Especially, I would like to thank Dr. Y.K. Ng for his comment on my thesis writing and Professor Vivian W.Y. Lui, who generously provided me many important experimental materials and advices on my project.

Most of all, I would like to thank my family, friends, especially Miss L.P.J., for their support and encouragement throughout my study.

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Abbreviations

5F	Ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid
AIF	Apoptotic inducing factor
BSA	Bovine serum albumin
Caspases	Cysteine aspases
COX-2	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGFR	Epidermal growth factor receptor
HNSCC	Head and neck squamous cell carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	Human papillomavirus
HR	High risk
IAP	Inhibitor of apoptosis protein
MEM	Minimum Essential Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
NP-40	Nonident P-40
PARP	Poly (ADP) ribose polymerase
PBS	Phosphate buffered saline
PsL	Pteris semipinnata L
PI	Propidium iodide
PMSF	Phenyl-methy-sulphonylfluoride
RHD	Rel Homology domain
RIPA	Radioimmunoprecipitation
SDS	Sodium dodecyl sulfate
TNF-α	Tumour necrosis factor-α
WB	Western Blotting

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Chapter One General Introduction

1.1 Background

Head and neck squamous cell carcinoma (HNSCC) has high incidence and mortality rate particularly in Southeast Asia and Eastern Europe (Franceschi et al. 1996) while laryngeal squamous cell carcinoma is the most common type of HNSCC worldwide (Jemal et al. 2006). It is more common in men than in women. When incidence and mortality are compared by gender, it was found that by 2005, the worldwide incidence of laryngeal carcinoma in male and female was 139,230 vs. 20,011 while the mortality was 78,629 in male and 11,327 in female (Parkin et al. 2005). Besides, the incidence of laryngeal carcinoma is also increasing. The global cancer statistics for laryngeal carcinoma reported 159,000 new cases and estimated 90,000 deaths in 2002. The latest report on cancer statistics in the United States of America estimated that 9680 men and 2570 women had laryngeal carcinoma as well as 2910 men and 760 women died due to the cancer in 2008 (Jemal et al. 2008).

Hong Kong is a high risk area for laryngeal cancer in terms of the incidence rate (Syrjanen 2005). In between 2000 and 2005, averagely 14 and 185 new cases of laryngeal carcinoma in women and men were reported respectively in each year (Hospital Authority: Hong Kong Cancer Registry). Due to the increasing incidence and mortality rate of laryngeal carcinoma, even though the patient population is not large worldwide or in Western Countries, more attention still need to paid for the development

of laryngeal carcinoma detection and treatment in Southeast Asia and in Hong Kong.

Multiple causative factors have been implicated in the development of laryngeal squamous cell carcinoma (SCC). Most of them are related to tobacco products and alcohol consumption, oral hygiene, health, lifestyle, sunlight exposure, age, and gender (Uobe et al. 2001). Among these, tobacco smoking, alcohol consumption and betel chewing are major risk factors of HNSCC. Heavy smokers and drinkers have a 17.6 fold and a 79.6 fold increased risk of laryngeal carcinoma respectively when compared with non-smokers and light drinkers (Franceschi et al. 1990).

However, a small proportion of HNSCC individuals do not have any history of tobacco or alcohol use, suggesting the presence of other possible causes of the cancers. One of the possible causes is the human papillomavirus (HPV) infection (anal, vaginal and vulvar), which is associated with an increased risk of developing tumours. Women with a history of *in situ* (Bjorge et al. 1995) or invasive cervical cancer (Boice et al. 1985; Rabkin et al. 1992) have a 2 to 4 fold increased risk of developing oral or laryngeal cancer.

1.2 Human papillomavirus infection at the larynx

Larynx is the most common site for HPV infection, which commonly causes laryngeal papillomatosis or recurrent respiratory papillomatosis (RRP) (Syrjanen 2005). While the HPV viral aetiology of cervical cancer is well-characterized (Walboomers et al. 1999), its role in the larynx was first described by Ullman (Ullman UV 1923). The supercoiled

viral DNA was first identified by Crawford and Crawford (Crawford and Crawford 1963). Later, the existence of different types of HPV was demonstrated and more than 90 HPV types have been characterized (Syrjanen et al. 1987; Herrero 2003; Chen et al. 2005). The virus types can be classified into high-risk (HPV 16, 18, 31 and 45), intermediate risk (HPV 33, 35, 39 and others) and low-risk (HPV 6 and 11) types, based on their epidemiologic association with cancer and benign epithelial hyper-proliferation (zur Hausen 1996). The observed roughly 80% prevalence of HPV in RRP is almost as high as in cervical cancer, which is a well-established HPV associated-disease (Derkay and Darrow 2000). While these lesions are basically benign, malignant transformation into laryngeal SCC does occur in a certain proportion of cases. Combining its high prevalence in RRP with its malignant potential, HPV may be associated with the development of laryngeal carcinoma.

The role of HPV in laryngeal carcinoma has been suggested by the detection of typical cytopathic effects of HPV in these lesions (Syrjanen and Surjanen 1981). The most convincing evidence that implicates HPV in laryngeal cancer is derived from studies demonstrating the presence of HPV DNA in cancer lesions. In a systematic review by Kreimer, 24% of the samples were reported to be HPV positive in 1435 larynx SCC specimens from 18 countries and 35 studies. Of the HPV positive larynx SCC, HPV 16 was the most common type with a 69.2% prevalence rate while HPV 18 accounted for a 17% prevalence rate (Kreimer et al. 2005). It is currently suggested that the high risk HPV 16 and 18 are responsible for a subset of laryngeal carcinoma although the reported HPV DNA detection rates varied widely (Syrjanen and Puranen 2000; Herrero 2003).

1.2.1 Biology of human papillomavirus

Human papillomavirus is a small non-enveloped DNA virus with a virion the size of ~55nm in diameter (Zheng and Baker 2006). HPV replicates and assembles exclusively in the nucleus of the host cell. All HPVs contain a double-stranded, circular DNA genome approximately 8kb in size that can be divided into three major regions: early, late and a long control region (LCR, a noncoding region). The genome of the early region occupies over 50% of the virus genome and encodes six proteins (E1, E2, E4, E5, E6 and E7). E1 and E2 are involved in the replication of the viral genome and interact with the host cell intermediate filament E4. The function of the E3 gene is unknown. E5, E6 and E7 are viral oncoproteins which induce cell immortalization and transformation. In particular, E6 and E7 inactivate two cellular tumor suppressor proteins p53 and pRb respectively (Garnett and Duerksen-Hughes 2006). Moreover, the E2 protein is a transcriptional repressor of E6 and E7 gene expression. As the integration of HPV DNA into the host genome results in deletion of part of the virus genome, loss of E2 was thought to result in the over-expression of E6 and E7 (Tan et al. 1994; Corden et al. 1999). The late region covers almost 40% of the virus genome and encodes L1 and L2 open reading frame for translation of a major (L1) and minor (L2) capsid protein. The LCR region, a segment of about 850bp, does not encode any protein but plays a role in the origin of replication as well as in multiple transcription factor binding sites. As the E6 and E7 proteins of the high risk (HR) HPV play a pivotal role in cell proliferation, viral replication and circumvent host-mediated apoptosis, many studies focus on the roles of these two proteins in carcinogenesis.

1.2.2 HPV E6 protein

E6 plays crucial roles in the HPV viral life cycle, as well as in cellular transformation and immortalization (Bedell et al. 1989). It is about 150 amino acids in size and is expressed in two forms: a full-length version of 16kDa and a truncated form about half that size corresponding to the N-terminal half of the full-length protein (Mantovani and Banks 2001). Two zinc fingers are composed of four Cys-X-X-Cys (X representing any amino acid) motifs which are responsible for E6 function (Cole and Danos 1987; Barbosa et al. 1989). HR HPV E6 protein contains a highly conserved five amino acids C-terminal domain which mediates direct binding to PDZ proteins. PDZ domain proteins serve critical roles in various molecular processes including cell polarity and signal transduction. The binding of E6 and PDZ proteins is clearly important for HPV associated carcinogenesis, because deletion of the relevant domain inhibits E6 driven transformation in rodent cells (Kiyono et al. 1997) and epithelial hyperplasia in mice (James et al. 2006). Further, the expression of the E6 protein results in activation of the telomerase reverse transcriptase (TERT) promoter allowing for increased telomerase activity and extension of the cellular life-span through elongation of the telomeric repeats (Klingelhutz et al. 1996).

The first identified and best characterized E6 interacting partner is tumour suppressor p53 (Werness et al. 1990). The p53 transcription factor is the most frequently inactivated tumour suppressor gene in human cancer and is involved the control of cell proliferation in response to stress. Under normal physiological conditions, the p53 protein level is very low. In the early stages of HR HPV infection, E7 oncoprotein induces a significant

increase in cell proliferation as a result of its interaction with the protein retinoblastoma, which triggers the expression of p53 (Longworth and Laimins 2004). In the absence of E6, the rise in p53 levels would lead to cell cycle arrest and/or apoptosis. However, the case is completely different in the presence of E6. E6 binds to p53 with the aid of E6-associated protein (E6-AP) ligase and prevents p53 from inducing growth arrest and apoptosis by subjecting it to ubiquitin-proteasome degradation pathway (Huibregtse et al. 1991). Moreover, E6 also precludes the growth-suppressive activities of p53 by cytoplasmic sequestration and by transcriptional suppression of its target genes (Mantovani and Banks 2001).

There are a number of proteins other than p53 targeted by E6. These proteins involve in regulation of transcription (Zimmermann et al. 1999), DNA replication (Gao et al. 1999), apoptosis and immune evasion (Gross-Mesilaty et al. 1998; Thomas and Banks 1998), epithelial organization and differentiation (Tong and Howley 1997), cell-cell adhesion and proliferation control (Thomas et al. 2001), and DNA repair (Srivenugopal and Ali-Osman 2002).

Of the proteins involved in apoptosis, it was found that E6 inhibits Bak-mediated apoptosis by directly binding to Bak, an interaction which is conserved in high risk and low risk HPVs (Thomas and Banks 1998; Thomas and Banks 1999). In our laboratory, E6 was found to inhibit TNF-mediated apoptosis by reducing the expression of Bak and increasing Bcl-2 expression without significantly affecting the expression of caspase-3 and -8 in laryngeal cancer cells (Du et al. 2004).

1.2.3 HPV E7 protein

HR HPV E7 protein, small dimerized phosphoprotein, is approximately 100 amino acids in size with three conserved regions: CR1, CR2 and CR3, which are critical for viral oncogenic activities (Wise-Draper and Wells 2008). There is a Leu-X-Cys-X-Glu motif within CR2 that mediates the binding with retinoblastoma (Rb) protein family, and is also necessary for viral DNA maintenance during the infectious cycle (Dyson et al. 1989; Munger et al. 1989; Datto et al. 1995). The CR3 region located at the C-terminus consists of a metal binding domain composed of two Cys-X-X-Cys motifs separated from each other by 29 amino acids. The C-terminal zinc-binding regions are important for E7 protein structure including dimerization (Clemens et al. 1995; Clements et al. 2000), intracellular stabilization (Edmonds and Vousden 1989; Phelps et al. 1992), as well as for the formation of high-molecular mass oligomers with apparent chaperone holdase activity (Alonso et al. 2006). Many cellular interacting partners for E7 have been identified, but the biological significance for most of the observed interactions remains to be explored.

What have been intensively studied are the Rb, p107 and p130, members of the retinoblastoma family, which are the targets of the E7 protein (Dyson, Howley 1989; Berezutskaya *et al.* 1997). Both low and high risk E7 can interact with Rb, however, the relative Rb binding affinities are 10 fold higher in high risk E7 than in low risk E7 (Munger et al. 1992). Rb has an important role in the suppression of cellular proliferation (Cobrinik 2005), stimulation of differentiation and senescence (Deshpande et al. 2005; Dimri 2005), cell survival (Chau and Wang 2003) and the maintenance of

stem cell quiescence (Ruiz et al. 2004). Normally, Rb forms a complex with histone deacetylase (HDAC) and binds to the E2F transcription factor in the G1 phase of the cell cycle. This prevents E2F from transactivating genes that are necessary for proliferation until the cell enters the S phase. However, the mechanism is different when E7 is expressed in cells. E7 binds to the complex of Rb and HDAC and relieves their repression of E2F, resulting in the constitutive activation of repression genes. Consequently, E7 provokes the cell to re-enter the S phase, where cellular replication factors required for viral replication are activated.

E7-Rb interactions are not sufficient for the abrogation of cell cycle arrest. Such activity also requires the binding of E7 and the proteins involved in cell cycle control such as cyclin-dependent kinase cdk2 and cyclin A (Tommasino et al. 1993), proteins regulating transcription such as the TATA box-binding protein and transcription factors AP1 (Antinore et al. 1996), and proteins for other cellular functions such as TAF-110 and TBP (Mazzarelli et al. 1995).

In addition to its role in cell proliferation and viral replication, E7 also regulates apoptosis. However, it has dual effects on cellular apoptotic pathways. It was reported that the expression of E7 in fibroblasts delayed Fas-mediated apoptosis and prevented TNF-mediated apoptosis by suppression of caspase-8 activation (Thompson et al. 2001). On the other hand, the majority of studies suggest that E7 serves a pro-apoptotic role. In a recent study, E7 was shown to inhibit TNF-mediated apoptosis in keratinocytes by up-regulating the expression of the inhibitor of the apoptosis protein c-IAP2 (Yuan et al. 2005). Moreover, E7 has been shown to sensitize mouse lymphoma cells (JD3) to

IFN-α-induced apoptosis (Thyrell et al. 2005), co-express with p21 and induce apoptosis in U2Os osteosacroma cells (Kaznelson et al. 2004), and over-express in genital keratinocytes which induces spontaneous cell death and sensitization to TNF-mediated apoptosis (Stoppler et al. 1998).

1.3 Apoptosis

One way to kill cancer cells is by inducing the process of programmed cell death called apoptosis.

Apoptosis in cancer cells can be induced by a number of triggers as diverse as hypoxia, a shortage of nutrients or growth factors, and radiotherapy or chemotherapy. Apoptosis is different from necrosis, which is usually immunologically harmful due to the sudden release of proinflammatory mediators (Vakkila and Lotze 2004). Necrosis is morphologically characterized by swelling of the cytoplasm, leading to the rupture of the plasma membrane, and the release of swollen and damaged organelles. Necrotic cell death often causes the release of proinflammatory cytokines, such as interleukin-8, IL-10, TNF-α (Fadok et al. 2001), or of terminal mediators of inflammation, such as high-mobility group box1 (Wang et al. 1999; Scaffidi et al. 2002).

During the early stage of apoptosis, cell shrinkage and pyknosis are the two visible morphological changes under the light microscopy (Kerr et al. 1972). With cell shrinkage, the cells become smaller in size; the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and is the most

characteristic feature of apoptosis. Apoptotic cell appears as a round or oval masses with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies that consist of cytoplasm with tightly packed organelles. Because the apoptotic bodies are subsequently phagocytosed by macrophages and degraded within phagolysosomes, there is no inflammatory reaction associated with the process of apoptosis. The cells are rapidly phagocytosed by surrounding cells, preventing secondary necrosis, and the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok 2000; Kurosaka et al. 2003).

Biochemically, apoptosis is initiated by the cleavage of pro-caspase-3. Cleaved caspase-3 induces DNA fragmentation, cross-linking of proteins, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Hengartner 2000).

In addition to two major apoptotic pathways, the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathway), there is another pathway induced via either granzyme B or granzyme A. All three pathways converge on the same terminal or execution pathway. The granzyme A pathway activates a parallel, caspase-independent cell death pathway through the damage in single stranded DNA (Martinvalet et al. 2005). Caspases are widely expressed in an inactive proenzyme form and have proteolytic activity to cleave proteins at aspartic acid residues, involving recognition of neighboring amino acids. Ten major caspases have been identified and are broadly categorized into initiators (caspase-2,-8,-9,-10), executioners (caspase-3,-6,-7) and inflammatory

caspases (caspase-1,-4,-5) (Cohen 1997; Rai et al. 2005). Extensive protein cross-linking is another characteristic, achieved through the expression and activation of tissue transglutaminase (Nemes et al. 1996). DNA breakdown by Ca^{2+}-and Mg^{2+}-dependent endonucleases also occurs, resulting in DNA fragments with 180 to 200 base pairs. Expression of cell surface markers results in the early phagocytic recognition of apoptotic cells by adjacent cells and phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the movement of phosphatidylserine from the inner membrane to the outer layer (Bortner *et al.* 1995).

1.3.1 Apoptosis signaling pathways

Two major pathways are involved in the signaling of apoptosis: the death receptor mediated extrinsic pathway and the non-receptor mediated intrinsic pathway.

The extrinsic pathway initiates apoptosis through transmembrane receptor-mediated interactions. These involve death receptors that are members of the TNF receptor gene superfamily (Locksley et al. 2001). There is a death domain, located in the cytoplasm, responsible for transmitting the death signal from the cell surface to the intracellular signaling pathways (Ashkenazi and Dixit 1998). To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Chicheportiche et al. 1997; Ashkenazi and Dixit 1998; Peter and Krammer 1998; Suliman et al. 2001; Rubio-Moscardo et al. 2005). FasL/FasR and TNF-α/TNFR1 are the best characterized models to elaborate the extrinsic pathway. The binding of Fas ligand and receptor results in the binding of adapter protein

Fas-associated death domain (FADD), while the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TNF-receptor-associated death domain (TRADD) with recruitment of FADD and receptor inactive protein (RIP) (Hsu et al. 1995; Wajant 2002). After that, FADD associates with pro-caspase-8 via dimerization of the death effector domain, forming a death-inducing signaling complex (DISC), leading to the auto-catalytic activation of pro-caspase-8 and the apoptosis is triggered (Kischkel et al. 1995).

The intrinsic pathway involves a variety of non-receptor-mediated stimuli, including growth factors, hormones, cytokine, radiation, toxins, hypoxia and viral infections, that produce intracellular signals targeting mitochondrial. Upon stimulation, the inner mitochondrial membrane is changed resulting in opening of the mitochondrial permeability transition (MPT) pore and loss of the mitochondrial transmembrane potential followed by the release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens et al. 2004). The first group of proteins includes cytochrome c, second mitochondrial activator of caspases (Smac), direct IAP (inhibitors of apoptosis proteins) binding protein with low PI (DIABLO) and serine protease HtrA2/Omi, which binds and activates Apaf-1 as well as pro-caspase-9 and inhibits IAP activity (Du et al. 2000; Schimmer 2004; Garrido et al. 2006). The release of a second group of proteins is a late event that occurs after the cell is committed to die. It includes pro-apoptotic proteins, apoptotic inducing factor (AIF), endonuclease G and caspase activated DNase (CAD). AIF translocates into the nucleus and cleaves the DNA into 50-300kb fragments and condensates peripheral nuclear chromatin (Joza et al. 2001). Endonuclease G can cleave nuclear chromatin to

produce oligonucleosomal DNA fragments (Li et al. 2001). Both AIF and endonuclease G are caspase-independent while CAD is cleaved by caspase-3 in order to cut the DNA and condensate the chromatin (Enari et al. 1998).

The control and regulation of these apoptotic mitochondrial events occurs through members of the B-cell lymphoma protein 2 (Bcl-2) family proteins which are regulated by tumor suppressor protein p53 (Cory and Adams 2002). To date, 25 Bcl-2 family members, either pro-apoptotic or anti-apoptotic, govern the mitochondrial membrane permeability. It is believed that Bcl-2 family proteins are mainly activated through the regulation of cytochrome C release from the mitochondria via alteration of mitochondrial membrane permeability.

Both the extrinsic and intrinsic pathways end at the point of execution, considered as the final pathway of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, -6 and -7 function as executioner caspases responsible for cleaving various substrates including cytokeratins, poly(ADP-ribose) polymerase (PARP), the plasma membrane cytoskeletal protein alpha fodrin, the nuclear mitotic apparatus protein (NuMA) and others, that ultimately cause the morphological and biochemical changes (Slee et al. 2001).

1.4 Transcription factor: Nuclear factor - κB

The nuclear factor-kappaB (NF-κB) pathway is known to be one of the most important cellular signaling transduction pathways involved in apoptosis. Also, NF-κB regulates other biological functions such as immune function, differentiation, inflammation, stress response, and cell survival. NF-κB is constitutively activated in many human cancer cells including pancreatic caner (Li et al. 2004), breast and prostate cancer (Nakshatri et al. 1997; Shukla et al. 2004), gastric carcinoma and head and neck squamous cell carcinoma (Allen et al. 2007; Levidou et al. 2007), suggesting that NF-κB activation plays an important role in cancer development. Thousands of experimental studies have shown that down-regulation of NF-κB activity by natural and synthetic inhibitors suppresses the development of carcinogenesis, inhibits the growth of cancer cells, and induces apoptosis with alteration of gene expression which is critical for the control of cancer progression and cancer cell survival. Therefore, it is becoming obvious that inhibition of NF-κB activity is highly desirable in the prevention and treatment of cancer and, as such, the chemopreventive agents that are known to down-regulate the activity of NF-κB could potentially inhibit the development and progression of cancers.

1.4.1 Overview of the NF-κB signaling pathway

In mammalians, transcription factor NF-κB is a five member family consisting of homo- and heterodimers, p65 (RelA), RelB, c-Rel, p50 and p52, and respectively encoded by the *rela, relb, rel, nfkb1* and *nfkb2* genes (Gilmore 2006). These proteins are characterized by the presence of a conserved 300 residue homologous domain near the N

terminus (Baldwin 1996; Ghosh et al. 1998). This domain, called the Rel Homology domain (RHD), is responsible for dimerization, DNA binding, inhibitor binding and nuclear localization (Huxford et al. 1999). On X-ray crystal structure analysis, the RHD is composed of two-folded domains linked by a short polypeptide (Ghosh et al. 1995).

In the resting status, NF-κB is bound by inhibitor κB (IκB) proteins and hence sequestered in the cytoplasm as an inactive form. There are seven IκB family members, IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p105 (p50 precursor protein) and p100 (p52 precursor protein), which are Ankyrin Repeat Domain (ARD) containing super-family. The IκB kinase (IKK) complex (containing two kinase subunits: IKKα and IKKβ; one regulatory subunit: IKKγ) is responsible for the phosphorylation of IκB proteins. Two major signaling pathways have been characterized for the activation of NF-κB (Bonizzi and Karin 2004). In the canonical (classic) pathway of NF-κB activation, for example, upon stimulation by the proinflammatory cytokine tumor necrosis factor α (TNFα), signaling pathways lead to activation of the IκB kinase β (IKK) complex, which then phosphorylates IκB proteins on two N-terminal serine residues. In the non-canonical (alternative) pathway, IKKα is activated by NF-κB inducing kinase and phosphorylates the p52 precursor protein p100 (Senftleben et al. 2001). Phosphorylated IκBs are recognized by the ubiquitin ligase machinery, leading to the process of polyubiquitination and subsequent degradation, or in the case of p100, by proteasome (Karin and Ben-Neriah 2000). The NF-κB dimers are free to translocate to the nucleus and bind to specific sequences in the promoter or enhancer regions of target genes. Activated NF-κB can then be down-regulated through multiple mechanisms including negative feedback by newly synthesized IκB proteins which bind to and export NF-κB to

the cytosol.

Another pathway that can lead to NF-κB activation is based on activation of casein kinase 2 (CK2), instead of IKK, which induce IκBα degradation through the phosphorylation of carboxy-terminal sites. However, this pathway has only a minor role in physiological NF-κB activation. It might contribute to skin carcinogenesis because it is activated by ultraviolet radiation (Kato et al. 2003).

1.4.2 Regulation of NF-κB signaling

Given NF-κB's critical involvement in so many physiological responses, the regulation of NF-κB signaling is extremely important in response to diverse stimuli that can trigger distinct cellular responses in particular physiological contexts.

Without stimulation, NF-κB transcription factor exists in either homodimer or heterodimers in cytoplasm. The structure for dimerization, which is responsible for different stimuli, is regulated by the folding of amino acid side chains. The C-terminal Ig-like domain of approximately 100 amino acids within the RHD is completely responsible for dimer formation. In most NF-κB dimers, but not the RelB homodimer, each monomer contributes symmetrical β-strand elements that pack against each other to form a β-sheet dimer interface (Huang et al. 1997). The amino acid residues at the dimer interface are highly conserved and contribute to dimerization by the positive and negative energetic interaction across each member of NF-κB proteins (Hart et al. 2001). From studies of many cell types, the p50: RelA heterodimer is more abundant than the

RelA:RelA homodimer, with abundance of the cRel:cRel homodimer and p50:cRel heterodimer being intermediate. However, relative expression levels of NF-κB proteins and the abundance of different dimers are dependent on the cell type and cellular context in history (Hoffmann et al. 2006).

On stimulation, two major pathways are triggered, leading to different responses. There is a variety of stimuli that can activate the classical pathway: pro-inflammatory stimuli and genotoxic, including TNF and interleukin 1(IL-1) (Osborn et al. 1989); bacterial cell-wall components, such as lipopolysaccharide (LPS) (Sen and Baltimore 1986); viruses, such as Rabies virus (Nakamichi et al. 2005); and DNA damaging agents, such as doxorubicin. On the other hand, the alternative pathway can be induced by the ligation of certain groups of TNF family members including LTβR and B cell activating factor receptor (BAFFR) (Claudio et al. 2002).

Although both pathways can affect tumor development (the role of NF-κB on tumor progression will be discussed in next section), most of our current knowledge relates to the pro-carcinogenic functions of the classical pathway. In alternative pathways, the IKKα subunit has a negative-regulatory role by phosphorylating the NF-κB subunits RelA and cRel on sites that accelerate their nuclear turnover, leading to termination of the NF-κB-mediated gene-induction response. (Lawrence et al. 2005)

In response to diverse stimuli, different NF-κB dimers are activated to translocate to the nucleus and to bind to the specific target genes. The binding between NF-κB and target genes is tightly controlled since it directly links to the consequent cellular response. The

DNA sequences that specifically bind to NF-κB dimers are collectively known as κB sites. Most κB sites appear to be 10bp in length with the consensus sequence 5'-GGGRN W YYCC-3' (where R represents a purine base, N represents any base, W represents an adenine or thymine and Y represents a pyrimidine base) (Sen and Baltimore 1986; Chen and Ghosh 1999). The conformations of the κB DNA sites exhibit global structural likeness in their protein-bound forms. The DNA is slightly bent towards the major groove and the extent of bending is directly correlated to the length of the central tract of AT base pairs. The AT-rich sequence is known to possess a higher propensity for bending, with longer stretches being more prone to bending. This region of the DNA also makes far fewer direct contacts with the protein and the bending seems to facilitate NF-κB binding at the flanking recognition sequences (Huang et al. 2005). Both monomers of the NF-κB dimers will bind to the DNA sequences with different numbers of bp. Each NF-κB has some preference for a specific set of κB sites and some NF-κB dimers bind to most κB sites with reasonably high affinities. Through the X-ray structures of the different combinations of NF-κB dimers, it is revealed that the p50 and p52 subunits bind to the 5bp 5'-GGGRN-3' half-site of the consensus sequence and that the RelA, cRel and RelB subunit prefers the 4bp 5'-YYCC-3' half-site (Chen et al. 2000). The NF-κB interactions with its cognate DNA binding sites are very transient and have different affinities, implying a fast dissociation rate constant. In addition, the transcriptional control involves the coordinated function of many proteins, including DNA-bound transcription factors, co-activators and co-repressors in the context of nucleosomal chromatin. It has been shown that large number of proteins located on DNA regulatory sequences, protein-protein interactions, are likely to be as important as transcription factor-DNA interactions in determining transcriptional specificity.

The transcriptional regulation by the NF-κB signaling module is highly sophisticated and requires integration of different mechanisms that can generate specificity: dynamically control each member of the family of NF-κB dimers, interact with NF-κB and κB sites, and alternate conformations of the resulting NF-κB-κB DNA complex allowing for alternate co-activator interactions.

1.4.3 Roles of NF-κB in cancers

NF-κB is a key regulator for the expression and function of a number of genes involved in tumourigenesis.

NF-κB has dual roles in the regulation of apoptosis in cancer cells. In RelA-/- knockout mice, there is massive hepatic apoptosis (Beg et al. 1995). Liver apoptosis has been shown to be TNF-α signal dependant in the developing liver, as crossing RelA-/- mice with either TNF-α-/- or TNFR-/- mice rescued this liver phenotype (Doi et al. 1999; Alcamo et al. 2001). Activation of NF-κB can abrogate TNF-α apoptotic activity because NF-κB targets genes encoding for inhibitors of caspase activation and apoptosis. Moreover, NF-κB also regulates the anti-apoptotic proteins including IAP1 and 2, X-linked IAP, survivin, cellular fas-associated death domain-like IL-1β-converting enzyme inhibitory protein (cFLIP), Bcl-xL, A1, TNF receptor associated factor 1 (TRAF1) and TRAF2 (Kucharczak et al. 2003). IAPs (c-IAP1, c-IAP2, and XIAP) suppress the apoptosis induced by both extrinsic and intrinsic pathways through direct inhibition of effector caspases (caspases-3, -6, and -7) (Deveraux et al. 1998). C-FLIP shares a high level of homology with pro-caspase-8 without catalytic activity. On

induction, c-FLIP associates with TNFR to compete with and block caspase-8 activation (Kreuz et al. 2001). Bcl-xL and A1 prevent the release of cytochrome c and subsequent caspase-9 activation (Kucharczak et al. 2003). TRAF proteins serve to amplify NF-κB activation and to interfere with the caspase cascade at the TNFR1 level (Luo et al. 2005). In addition, NF-κB may inhibit prolonged c-Jun-N-terminal kinases (JNK) activation and the accumulation of reactive oxygen species (ROS) in order to block apoptosis. In fact, the effect of NF-κB on apoptosis is mostly dependent on the stimulus, cell-type, and the involved subunits (Sharma and Narayanan 1996). NF-κB contributes to cell death by transcriptionally up-regulating its pro-apoptotic target genes such as Fas/CD95, FasL, death receptor 4 (DR4) and DR5 (Wiener et al. 2004). Another pro-apoptotic mechanism involves p100, carrying death domain in the C-terminus, which mediates the recruitment of p100 to death receptors, such as TNFR1 and Fas, leading to caspase-8 activation before apoptosis (Wang et al. 2002).

NF-κB plays an important role in tumor invasion and metastasis by regulating angiogenic factors such as matrix metalloproteinase 2 (MMP2), MMP9 and serine protease urokinase-type plasminogen activator (uPA) (Novak et al. 1991; Rangaswami et al. 2004). Transfection of high metastatic human melanoma variant cells with a dominant-negative mutant IκBα shows inhibition of tumor growth and prevents lung metastasis in nude mice (Huang et al. 2000). In addition, NF-κB also regulates the expression of intercellular adhesion molecule 1 and vascular cell-adhesion molecule 1, the secretion of chemokines (monocyte chemoattractant protein-1, IL-8), growth factors (TNF, VEGF) and cyclooxygenase 2 (COX-2) and epidermal growth factor receptor (EGFR) (Loch et al. 2001).

NF-κB promotes cell cycle progression by regulating the expression of target genes involved in the cell cycle machinery such as cyclin D1, D2, D3, E and c-myc. The D family of cyclins in complexes with the catalytic subunits cyclin-dependent kinases (CDK4) and CEK6 are the main regulators required for entry and passage through G1 phase (Guttridge et al. 2000; Hinz et al. 2001; Hsia et al. 2002).

1.5 Pteris semipinnata L extract: ent-11-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F)

Natural product is an infinite source for remedies for long time and certain diet-derived substances can be used to prevent cancer or delay its onset has currently elicited considerable interest.

Many evidences have shown Chinese herbal medicine could be a potential anti-cancer drug by inducing differentiation, apoptosis, inhibiting angiogenesis, reversing multi-drug resistance through different mechanism (Ruan et al. 2006). Pteris semipinnata L, a Chinese traditional herb, was traditionally used to treat enteritis, hepatitis and snake bites. The natural chemical compound ent-11-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F), an ethanolic extract of PsL, has been shown to carry anti-tumor activity by inducing apoptosis.

In human gastric cancer, 5F induced DNA fragmentation and reduction of mitochondrial membrane potential, resulting in cell apoptosis through the translocation of Bax from cytosol to mitochondria in a p53-dependent manner (Liu et al. 2005A). In addition, it

was found that 5F induced thyroid cancer cell apoptosis in time- and dose- dependent manner by the activation of c-Jun N-terminal kinase and the rapid increase in intracellular reactive oxygen species levels (Liu et al. 2005B). Moreover, 5F could also induce apoptosis in human colon cancer HT-29 cells. It was demonstrated that B-cell lymphoma protein 2 (Bcl-2) or Bcl-xL were over-expressed in HT-29 cells leading to promotion of NF-κB activity and reduction of 5F-induced apoptosis. Furthermore, IκBα supper repressor, an NF-κB inhibitor, restored the apoptosis inducing ability of 5F even cells transfected with Bcl-2 (Chen et al. 2004). Such finding indicated NF-κB related pathway could be one of the apoptosis mechanism induced by 5F.

In cancer chemoprevention and chemotherapy, induction of apoptosis is a popular strategy by introducing the cancer drug either orally or injection. Since 5F can induce apoptosis as shown by many evidences, it can be employed as a potential apoptosis inducting drug in laryngeal cancer therapy. As mentioned in the previous section (1.2), HPV could promote anti-apoptosis. How 5F functions in HPV positive or negative laryngeal cancer cells is unknown. Moreover, NF-κB, one of 5F target in human colon cancer cells, is one of the most important apoptosis regulators. Because NF-κB activity was increased in laryngeal cancer (Du et al. 2003), its role in 5F-induced apoptosis might be pivotal.

1.6 Objectives

In this project, three laryngeal cancer cell lines, UMSCC11A, UMSCC12 and HEp-2, were employed. HEp-2 is HPV 18 positive while the others are HPV 18 negative. By

using these three cancer cell lines as models, two major objectives were investigated, including analysis of the apoptosis inducing effect of 5F in human laryngeal cancer cells with or without HPV18 infection and study the role of NF-κB in 5F-induced apoptosis in laryngeal cancer cells accompanied with the evidence shown by NF-κB specific inhibitor, Bay (11-7082) (Chopra et al. 2008).

Chapter Two Materials and Methods

2.1 Cell culture

Two human laryngeal cancer cell lines (UMSCC11A and UMSCC12) were kindly provided by T Carey from the University of Michigan. They were cultured in Minimum Essential Medium (MEM, GIBCO-BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100U/ml penicillin (GIBCO-BRL, Carlsbad, CA), 100μg/ml streptomycin (GIBCO-BRL, Carlsbad, CA), at 37°C in a humidified atmosphere of 5% CO_2 and 95% CO_2. The culture medium was changed once every two days.

Human laryngeal cancer cells, HEp-2 (American Type Culture Collection, ATCC), containing an integrated HPV18 genome, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO-BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100μg/ml streptomycin at 37°C in humidified atmosphere of 5% CO_2 and 95% CO_2. The culture medium was changed once every three days.

2.3 Cell proliferation analysis

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay that measures the cell growth and determines the cytotoxicity of a toxic agent. The assay is based on the reduction of yellow MTT that is converted into deep blue formazan crystals by mitochondrial reductase from viable cells. The crystals

are impermeable to the cell membrane and so accumulate in viable cells. A solubilization solution, such as dimethylsulphoxide (DMSO), is added to dissolve the insoluble purple crystal solution. The absorbance of this colored solution is quantified by measuring its absorbance at a wavelength of 570nm by spectrophotometer. The absorption maximum depends on the solvent employed.

The reducing reaction takes place in mitochondria when reductase is active. Therefore, the number of viable cells is directly proportional to the conversion of yellow MTT. By comparing the amount of formazan between the treatment group and control group, the effectiveness (and hence cytotoxicity) of an agent in causing cell death can be determined by generating a dose-response curve.

In our experiment, cells (UMSCC11A, UMSCC12 and Hep-2) were seeded at the density of 5 x 10^3/100μl/well in 96-well microtitration plates (NUNC) for 18 hours. A variety of concentrations of 5F (0.1% Dimethyl Sulphoxide (DMSO) (D2650, Sigma), 3.125, 6.25, 12.5, 25, 50, 100 and 200μg/ml), diluted by medium, were added and incubated for 24 or 48 hours after aspirating the old medium. Each group of concentrations were performed in triplicate wells and repeated three times. After different periods of incubation, the medium was aspirated, and then 100μl MTT (Sigma, St. Louis, MO) (diluted by phosphate buffered saline (PBS)) was added. The diluted MTT was added in each well and incubated for 3 hours at 37°C to allow viable cells to metabolize the MTT. The MTT was aspirated and the formazan crystals were dissolved by adding 100μl/well DMSO. The absorbance was measured at a wavelength of 570nm with reference to a wavelength of 630nm with a spectrophotometer (VICTOR^{3}TM

Multilabel Counter, PerkinElmer). Cell viability was presented as a percentage of the control group.

2.3 Western Blotting

Western Blotting involves several steps including protein extraction, protein quantification, gel electrophoresis, transferring protein, immunoblotting and developing.

2.3.1 Total protein extraction

Cells (5 x 10^5) were detached by warm Trypsin-EDTA (GIBCO-BRL, Carlsbad, CA) from the culture dish and washed twice with ice-cold PBS, then resuspended in 100µl radioimmunoprecipitation buffer (RIPA buffer: 1X PBS, 1% Nonident P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% Sodium dodecyl sulfate (SDS), pH7.6) together with the protease inhibitor cocktail (Roche) and 100µg/ml phenyl-methy-sulphonyl-fluoride (PMSF). The cells were then further disrupted and homogenized by passing them 20 times through a 21 gauge needle and then incubating them at 4°C for 45 minutes. After centrifugation for 10 minutes at 10000rpm at 4°C, the supernatant was recovered and stored at 4°C.

2.3.2 Nuclear and cytoplasmic protein extraction

Cells (10^6) were detached by warm Trypsin-EDTA from the culture dish and washed twice with ice-cold PBS. They were resuspended by up-and-down pipetting several

times with 100µl 1X Buffer A (10 x Buffer A stock: 100mM *4*-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH7.9, 100mM KCl, 100mM (ethylene diamine tetraacetic acid) EDTA; 1ml 1X Buffer A adding 10µl 100mM Dithiothreitol (DTT), 10µl protease inhibitor cocktail, 40µl 10% NP-40) in a 1.5ml eppendorf tube that was fixed in a rocking platform and shaken at 150rpm for 10 minutes in a cold room. After centrifugation for 3 minutes at 4°C at 15000 g, the supernatant (cytosolic fraction) was collected. The pellet was resuspended in 60µl 1X Buffer B (5X Buffer B stock: 100mM HEPES, pH7.9, 2M NaCl, 5mM EDTA, 50% glycerol; 147µl 1X Buffer B adding 1.5µl protease inhibitor cocktail and 1.5µl 100mM DTT) by vortexing it at the highest setting for 10 seconds or by using a Dounce homogenizer. Then the eppendorf tube was laid horizontally on the rocking platform and shaken at 200rpm for an hour. Finally, the nuclear fraction was obtained from the supernatant after centrifugation at 15000 g for 10 minutes at 4°C.

2.3.3 Quantification of protein concentration

The extracted protein (total protein, cytosolic protein and nuclear protein) was quantified by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). A standard curve was generated by serial dilution of bovine serum albumin (BSA, R396E, Promega, Madison WI, USA) with different lysis buffer (total protein: RIPA, Cytosolic fraction: Buffer A, Nuclear fraction: Buffer B) ranging from 0 – 1.4µg/ml. The assay was performed in a 96-well plate (Nunc, Denmark) by adding 5µl of standard and sample proteins into each well with a triplicate setting. After pre-treatment of 25µl Reagent A' (1ml Reagent A and 20µl Reagent S) for about 20 seconds, each standard and sample protein was added to

200µl Buffer B (dye reagent). After 15 minutes of incubation at room temperature, the protein concentration was determined by measuring the absorbance at a wavelength of 595nm with a spectrophotometer (Fluo Star Galaxy, BMG Labtechnologies Pty. Ltd).

2.3.4 Sodium dodecyl sulfate – polyacylamide gel electrophoresis (SDS-PAGE) and protein transfer

An aliquot of 25µg total protein (cytosolic protein: 30µg, nuclear protein: 10µg) was mixed with 6X SDS loading buffer (350mM Tris-HCl pH6.8, 600mM DTT, 30% glycerol, 0.012% bromophenol blue) and denatured by boiling for 5 minutes at 99°C. The denatured protein and protein marker (Full Range Rainbow Recombinant Protein Molecular Weight Marker, GE Healthcare) were resolved by SDS-PAGE electrophoresis.

After electrophoresis, the gel was equilibrated for 5 minutes with cold transfer buffer (25mM Tris, 192 mM Glycine, 20% methanol, pH8.3). The proteins were then transferred onto a Nitrocellulose membrane by using a Bio-Rad Mini Trans-Blot cell. The transfer sandwich was packed with sponge on the bottom half of a plastic cassette using filter paper, protein gel, filter paper and sponge again. Protein larger than 65kDa was transferred for 120 minutes, while protein smaller than 65kDa was transferred for 90 minutes at 100V at room temperature. To optimize the transfer condition, the transfer tank was surrounded by ice to slow down the increase of current. After transfer, the membrane with proteins was washed with TBST (20mM Tris-Cl, 137mM NaCl, 0.1% Tween-20, pH7.6) buffer for 5 minutes before immunoblotting.

2.3.5 Immunoblotting

Membranes were incubated with 5% non-fat milk in TBST for 2 hours at room temperature prior the incubation with diluted primary antibodies at 4°C overnight. After washing with TBST three times in 10minutes, the membranes were incubated with corresponding secondary antibodies diluted with 2% non-fat milk in TBST for 2 hours at room temperature. Once again, the membranes were washed with TBST three times in 10minutes. Chemiluminescent was developed by adding ECL or ECL plus reagent kit (Amersham Life Science Ltd., Buckinghamshire, UK) and exposed to X-ray films.

2.4 NF-κB Luciferase Assay

To study the transcriptional activity of NF-κB, a genetic reporter assay was applied in our model. Firefly Luciferase assay was employed, firstly because its reporter activity is available immediately on translation. Secondly, the assay sensitivity is high due to its light production having the highest quantum efficiency for chemiluminescent reaction with no background luminescence is found in the host cells. Thirdly, the measurement can be done within a few seconds.

In our experiment, pNF-κB Luciferase plasmid (generously provided by Professor Vivian Lui, Department of Clinical Oncology, the Chinese University of Hong Kong) was transiently transfected into three laryngeal cancer cell lines: UMSCC11A, UMSCC12 and HEp-2. In the pNF-κB Luciferase plasmid (clone map attached in appendix), 5 times repeat NF-κB responsive elements (GGGGACTTTCC) were cloned

into a pLuc-MCS vector which encodes Firefly Luciferase. Once NF-κB was activated by TNF-α, it translocates into the nucleus and binds to NF-κB responsive elements on the vector. The translated Firefly Luciferase protein, a monomeric 61kDa protein, catalyzes luciferin oxidation which generates high intensity light that is constant and stable for at least 1 minute.

To perform the transient transfection, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was employed and the experimental procedures were followed according to the protocol recommended by the manufacturer. Briefly, cells (cell no. of UMSCC11A:1.8 x 10^5/ml/well, UMSCC12: 2 x 10^5/ml/well, Hep-2: 1.5 x 10^5/ml/well) were seeded in a 12-well culture plate (Corning, USA) for 18hrs before transfection. 1μg pNF-κB Luciferase plasmid and Lipofectamine 2000 in an optimized ratio (plasmid to Lipofectamine 2000 ratio: UMSCC11A: 1:1.5, UMSCC12: 1:2, HEp-2: 1:3) were mixed with OPTI-MEM (Sigma, St. Louis, MO) for 5 minutes separately. The plasmid and Lipofectamine 2000 were then mixed by pipetting up-and-down and then incubated for 20 minutes prior to being added to each well. After incubation for 5 hours, the cells were recovered and the transfection mixture replaced with by fresh medium (with 10% FBS).

24 hours later, cells were treated with different concentrations of 5F in serum free medium for 2 hours. 20ng/ml TNF-α was added into the medium for 6hrs in an attempt to stimulate the NF-κB transcription activity. The reaction was stopped by removing the medium and washing the cells twice with ice cold PBS. 120μl lysis buffer (0.05% Triton X-100, 100mM Tris-HCl, pH7.8, 2mM EDTA, buffer components following the protocol of Professor Vivian Lui) was added into each well and the cells were extracted

by a cell scraper. After vortexing for 15 seconds, the lysate was stored at -80°C overnight for higher lysis efficiency. After centrifuging at 13000 rpm for 10 minutes at 4□, the supernatants were aspirated into a new tube. 20μl cell lysate together with 50μl Luciferase assay reagent (Promega, USA) were mixed in a 96-well plate and the light intensity was measured by a spectrophotometer (VICTOR^{3}TM Multilabel Counter, PerkinElmer). To normalize the Luciferase assay, the cell lysate was subjected to protein quantification as mentioned in the previous part. Each assay was performed in triplicate in each experiment and repeated three times.

2.5 Annexin V apoptosis assay

In an attempt to detect 5F-induced apoptosis, Vybrant™ Annexin V- fluorescein kit (Molecular Probes, Eugene, OR) was employed in our study.

Apoptosis, or programmed cell death, is controlled by an internally encoded suicide program, representing a critical role in normal development. Apoptosis is distinguished from necrosis by distinct morphological and biochemical features, including shrinkage of the cytoplasm and loss of membrane asymmetry, condensation and fragmentation of the nuclear chromatin. In normal viable cells, phophatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. If the cell undergoes apoptosis, PS translocates from the inner plasma membrane to the outer leaflet, leading to the exposure of PS on to the external cellular environment. Such a characteristic feature provides us with a specific way to label an apoptotic cell and to determine the number of apoptotic cells.

Annexin V, a human anticoagulant, is a 35-36kDa $Ca2^{+}$-dependent phospholipid-binding protein that has a high affinity for PS. Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer the leaflet. The Vybrant™ Annexin V- fluorescein kit contains recombinant annexin V conjugated to Alexa Fluor 488 dye (a good spectral to fluorescein – FITC) and the red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI cannot permeate viable and apoptotic cells, but can however stain necrotic cells with a red fluorescence by binding tightly to the nucleic acids in the cell. After staining a population of cells with annexin V and PI, apoptotic cells show green fluorescence (FITC) while dead cells show red (PI) and green fluorescence, and viable cells show little or no fluorescence.

In this project, three laryngeal cancer cell lines (UMSCC11A, UMSCC12, HEp-2) were treated with different concentrations of 5F (0.1% DMSO, 50, 75, 100μg/ml) for 16 or 24 hours. Cells (UMSCC11A: 3.7 x 10^5, UMSCC12: 3.8 x 10^5, HEp-2: 3 x 10^5) were seeded in 6-well plates for 18 hours before treatment with 5F. After incubation for different periods, both detached and attached cells were collected by trypsization and centrifuged for 5 minutes at 2000 rpm. The cell pellet was then resuspended and washed three times with ice-cold PBS. For each sample, a master-mix solution, including 100μl binding buffer (10mM HEPES, 140mM NaCl , 2.5mM $CaCl_2$, pH7.4), 5μl Alexa Fluor 488 Annexin V, 2μl of 100μg/ml PI working solution, was added to resuspend the cell pellet and then incubated for 15mins in the dark at room temperature. After the incubation period, 400μl of binding buffer was added and the sample kept on ice in the dark and analyzed by flow cytometry (FACScalibur, Becton Dickson, CA) and using Cell Quest software (Becton Dickinson, CA).

2.6 mRNA expression analysis

To perform mRNA expression analysis, three steps were involved, including RNA extraction, reverse transcription (RT), and polymerase chain reaction (PCR).

2.6.1 RNA extraction

Total RNA of 1 x 10^6 cells were extracted and purified by using PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen, CA). After the incubation period, the cells, either detached or attached to the culture plate, were collected by trypsinization and washed three times with ice-cold PBS. Cell pellets were then resuspended in 300µl RNA lysis solution (1ml lysis solution added 10µl 2-mercaptoethanol) and mixed thoroughly with one volume of 70% ethanol by vortexing. All the samples were then transferred to the RNA Spin Cartridge and purified by following the protocol of the kit. The concentration and quality of the extracted RNA was determined by using a UV- spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

2.6.2 Reverse Transcription

Reverse transcription was performed according to the protocol of a Reverse Transcription System (Promega, Madison, WI). 1µg RNA was diluted into 10µl by adding RNase-free water and incubated at 70°C for 10 minutes and then placed on ice to cool down. 10µl of reagent from a master-mix solution was added into to each RNA

sample. For each reaction, the components of the master-mix solution include: 2μl Reverse Transcription 10X Buffer, 2μl dNTP mixture (10mM), 4μl $MgCl_2$ (25mM), 1μl Random Primer, 0.5μl Recombinant RNasin Ribonuclease Inhibitor (1u/μl), 0.5 μl AMV Reverse Transcriptase (25u/μl). The reaction mixture was incubated at room temperature for 10 minutes before performing the RT reaction: 42°C for 60 minutes, 95°C for 5 minutes. The cDNA product was incubated at 4°C. Through out the whole experiment, the cDNA and RNA stock was kept at -80°C.

2.6.3 Polymerase Chain Reaction

For each sample used to perform PCR, 2μl cDNA was added to 18μl master-mix solution which contains the following reagents: 1.5μl $MgCl_2$ (25mM)(Promega), 0.5μl dNTP (10mM), 1μl Forward and Reverse Primer (10ρg/μl), 0.5μl Go Taq Fexi DNA Polymerase (Promega,USA), 2μl 5X Green Go Taq Fexi Buffer (Promega, USA), 11.5μl dH_2O. The PCR mixture was firstly denatured by undergoing incubation at 94°C for 5 minutes, and then 25 cycles were performed under the following cycling conditions: 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec; extra time for extension: 72°C for 10 minutes, and incubated at 4°C. The sequences of the primers that we used are listed below:

Primer sequence of HPV 18 E6:

Sense: 5' ATGGCGCGCTTTGAGGATCCA 3'

Anti-sense: 5' TTATACTTGTGTTTCTCTGCGTCG 3'

Primer sequence of HPV 18 E7:

Sense: 5' ATGTATGGACCTAAGGCACACT 3'

Anti-sense: 5' TTACTGCTGGGATGCACACCA 3'

The PCR products were separated by 2% agarose gel and calibrated by running with DNA MW marker 100bp ladder (Promega, Madison, WI). The ethidium bromide stained DNA was visualized by Gel Doc XR (Bio-Rad, Italy) and software Quality One.

2.7 Antibodies

Antibodies that were used are listed below:

Antibody	Manufacturer
P65	Santa Cruz Biotechnology, USA
P50	Santa Cruz Biotechnology, USA
Lamin B	Santa Cruz Biotechnology, USA
β-tubulin	Santa Cruz Biotechnology, USA
β-actin	Santa Cruz Biotechnology, USA
Bax	Santa Cruz Biotechnology, USA
IκBα	Cell Signaling Technology, USA
COX-2	Cayman, USA (kindly provided by prof. Lui)
Cyclin D1 (SP-4)	Thermo Scientific, USA (kindly provided by prof. Lui)
EGFR (activated)	BD, Bioscience, USA (kindly provided by prof. Lui)
Survivin	Cell Signaling Technology, USA (kindly provided by prof. Lui)
Mouse IgG-HRP	Santa Cruz Biotechnology, USA
Goat IgG-HRP	Santa Cruz Biotechnology, USA
Rabbit IgG-HRP	Santa Cruz Biotechnology, USA

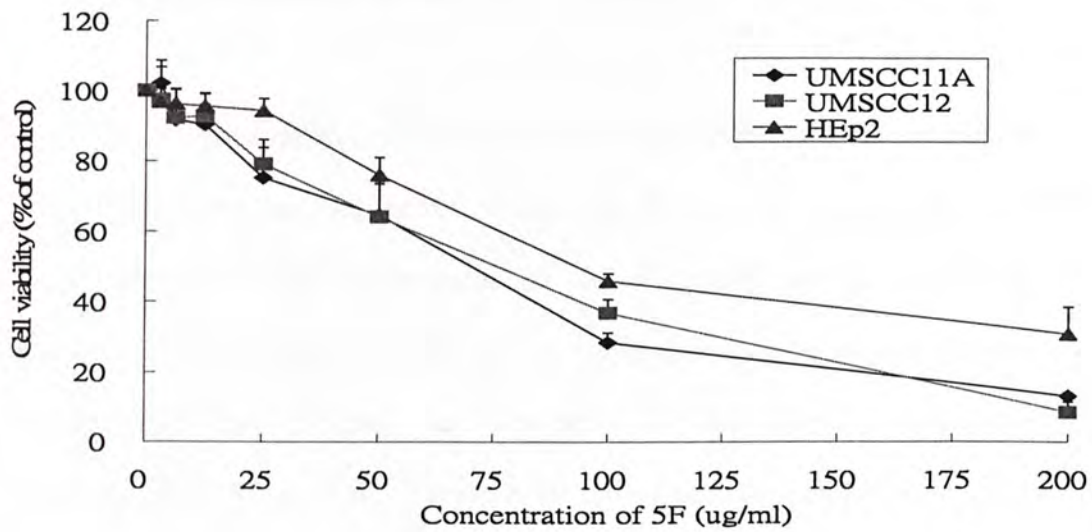
Chapter Three Results

3.1 Anti-proliferation effect of 5F on laryngeal cancer cells UMSCC11A, UMSCC12 and HEp-2 cells

We first investigated the effect of 5F on the growth of three laryngeal cancer cell lines: UMSCC11A, UMSCC12, and HEp-2 (HPV 18 positive). Cell proliferation was measured by MTT assay after 24 and 48 hours of treatment.

More viable cells were found in HEp2 cells (10% higher) than in UMSCC11A and UMSCC12 cells after 24 hours of treatment at different 5F concentrations (Figure 1). The percentage of viable cells in UMSCC11A was 65% at 50μg/ml 5F and decreased sharply to 28% at 100μg/ml. In UMSCC12, the viable cell percentage was 65% at 50μg/ml and 36% at 100μg/ml, while that in HEp-2 cells was 76% at 50μg/ml and 45% at 100μg/ml. After 48 hours of treatment, 5F showed a stronger inhibitory effect on the laryngeal cancer cells. But still, HEp2 was less susceptible to 5F, while UMSCC11A and UMSCC12 responded similarly to 5F. The percentage of viable cells in UMSCC11A was significantly ($P<0.05$) lower than in UMSCC12 and HEp-2 cells at 50μg/ml 5F at 48 hours. Our results show that 5F elicited a dose-dependent growth inhibition on all laryngeal cancer cell lines tested at 24 and 48 hours when compared to the control (0.1% DMSO). Through the dose-response curves at 24 and 48 hours, IC50 values of laryngeal cancer cells were determined: 50μg/ml for UMSCC11A and UMSCC12, and 75μg/ml for HEp-2.

A



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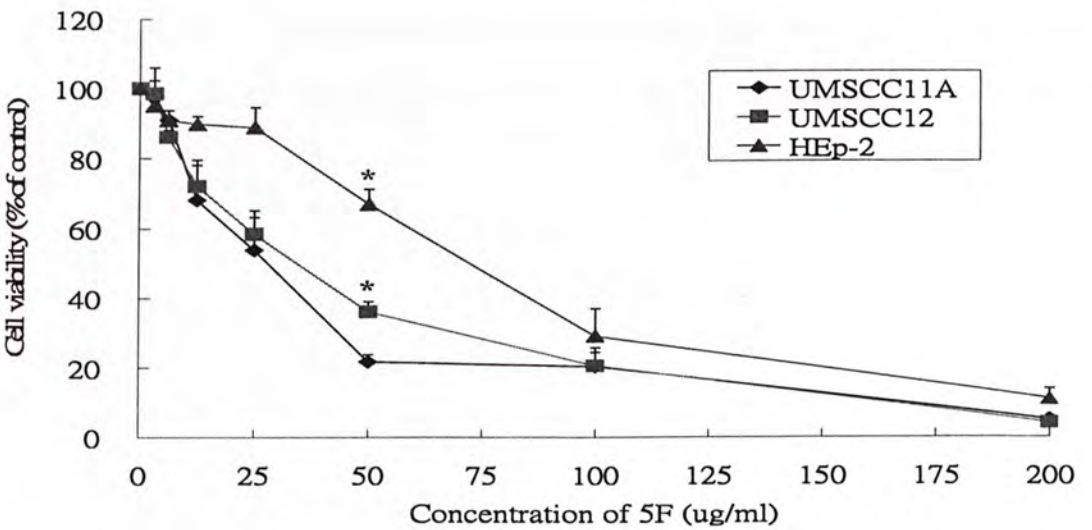
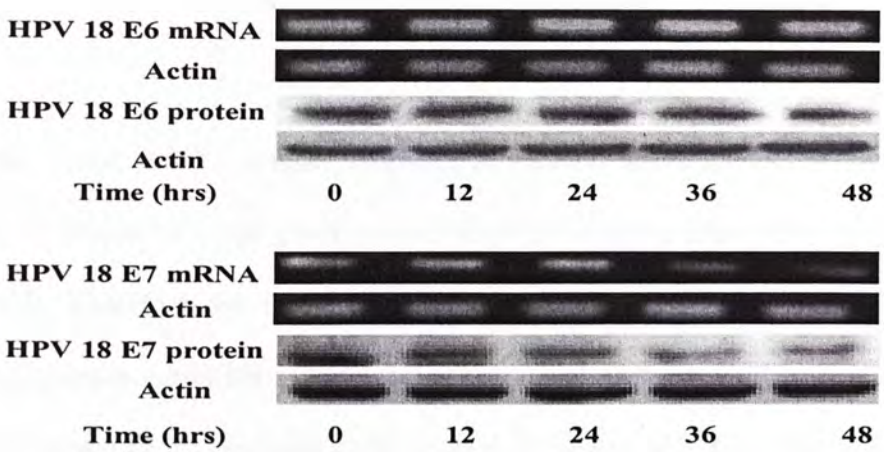


Figure 1.
5F inhibits cell proliferation in laryngeal cancer cell lines. UMSCC11A, UMSCC12 and HEp-2 were treated with different concentrations of 5F (0.1% DMSO, 3.125, 6.25, 12.5, 25, 50, 100, 200μg/ml) for 24 hours (A) or 48 hours (B). The MTT assay was performed as described in materials and methods. Values shown represent the mean of three separate experiments with each experiment performed in triplicate (* $P < 0.05$).

3.2 Suppression by 5F in HEp-2 of mRNA and protein expression levels in HPV18 E7 while the expression levels of HPV 18 E6 was not altered.

Since HEp-2 carries HPV18, which promotes cell growth in cervical cancer (Fujii et al. 2006), HPV18 may account for the resistance to the anti-proliferation effect of 5F on HEp-2. HPV18 encodes oncoproteins E6 and E7, which are responsible for viral replication, cell proliferation and for the prevention of host cell-mediated apoptosis. We postulated E6 and E7 may be responsible for the HEp-2 resistance to the anti-proliferation effect of 5F. Therefore, we investigated their steady-state mRNA and protein levels by semi-quantitative RT-PCR and Western blotting respectively after 5F treatment. It was found that neither the mRNA nor protein expression levels of E6 were altered after 5F treatment (Figure 2). In contrast, 5F suppressed both mRNA and protein expression levels of E7 in a time-dependent manner. Therefore, we suggest that the altered E7 oncoprotein levels in response to 5F may not be responsible for the HEp-2 resistance to 5F.

A



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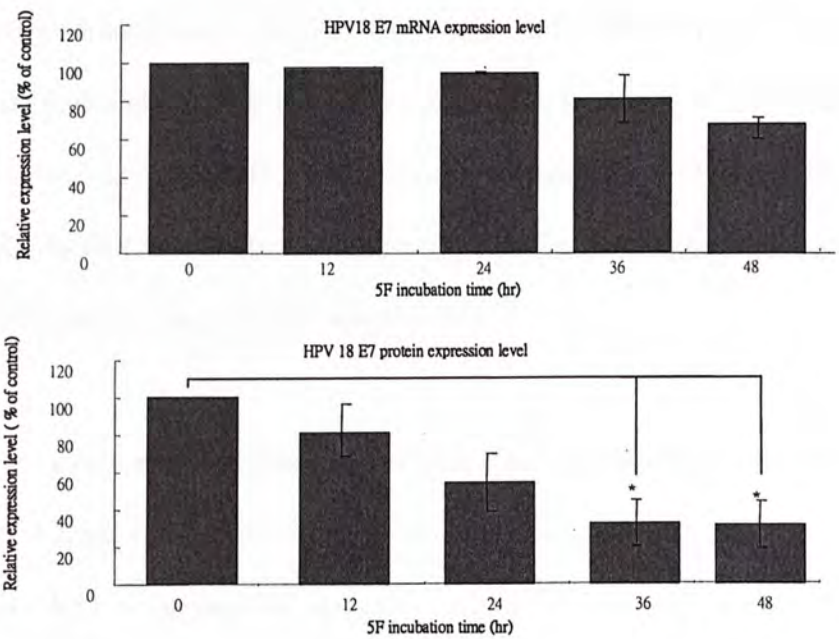


Figure 2.

5F down-regulated the mRNA and protein expression of HPV18 E7 but not the mRNA and protein expression of E6. HEp2 cells, which are positive for HPV18, were exposed to 75μg/ml 5F for different periods (0, 12, 24, 36 and 48 hours). Total RNA was extracted from 5F-treated cells and the full length of HPV18 E6 and E7 were amplified. The HPV18 E6 and E7 proteins were detected by Western blotting. The mRNA and protein expression levels of E6 were not altered, while that of E7 was down-regulated by 5F in a time-dependent manner (A). The relative expression level of E7 mRNA and protein were quantified by densitometer (B) (* $P < 0.05$).

3.3 Quantification of 5F-induced apoptosis in laryngeal cancer cells by Annexin V assay

It has been shown that 5F induces apoptosis in various cancers including colon cancer cells, thyroid cancer cells and gastric cancer cells (Chen, Liang 2004; Liu, Ng 2005; Liu, Chen 2005). Therefore, we further investigated whether apoptosis contributed to the 5F-induced growth inhibition of laryngeal cancer cells. Annexin V-PI co-labeling assay was used to quantify the apoptotic cells induced by 5F in UMSCC11A, UMSCC12 and HEp-2 cells. In the flow cytometry data obtained after the Annexin V labeling assay, the lower right quadrant (Ann V +ve/PI –ve) represents the distribution of early apoptotic cells (Figure 3, 4 and 5) while the upper right quadrant (Ann V +ve/PI +ve) represents the late apoptotic and dead cells. In this experiment, only the early apoptotic cells in the lower right quadrant were counted. The apoptotic cells in the upper right quadrant were excluded because they may include necrotic cells.

Following 5F treatments, apoptosis occurred in a dose-dependent manner in all the cell lines tested (Figure 3, 4 and 5). After 16 hours of treatment with 50, 75 and 100μg/ml, the corresponding percentages of apoptotic cells in UMSCC11A were 9.05%, 10.16% and 11.11% respectively. Similarly, after 24 hours treatment, the percentage of apoptotic cells increased from 16%, 17.64% to 22.44% under the same concentration at 16hrs, indicating that the degree of apoptosis was more significant with longer incubation time. However, in HEp-2, the apoptotic cells induced by 16 hours treatment of 100μg/ml 5F were only 2% higher than the vehicle control. Although more cells underwent apoptosis following a prolonged incubation time (24 hours), the level of apoptosis in HEp-2 was

significantly lower than that of UMSCC11A ($P<0.01$) and UMSCC12 under the same concentrations of 5F (Figure 6). Such differences might be due to the anti-apoptotic characteristic of HPV18 in HEp2. Comparing the two HPV18 negative cells, the percentage of apoptosis in UMSCC11A was statistically higher ($P<0.05$) than that in UMSCC12 when the dosage of 5F was 50 μg/ml at either 16 or 24 hours. Our results demonstrated that apoptosis is induced by 5F in laryngeal cancer cells UMSCC11A and UMSCC12, but at a lower degree in HEp-2.

3.4 Morphological changes in laryngeal cancer cells induced by 5F

Changes in cell morphology by 5F were observed under the microscope. When compared with the control group, 5F caused more shrinkage and detachment of the laryngeal cancer cells (Figure 7). Such morphological changes represented some of the apoptotic characteristics in laryngeal cancer cells. The degree of such morphological variations was proportional to the concentrations of 5F added to the cells. The scale of cell shrinkage and detachment was again smaller in HEp-2 than UMSCC11A or UMSCC12.

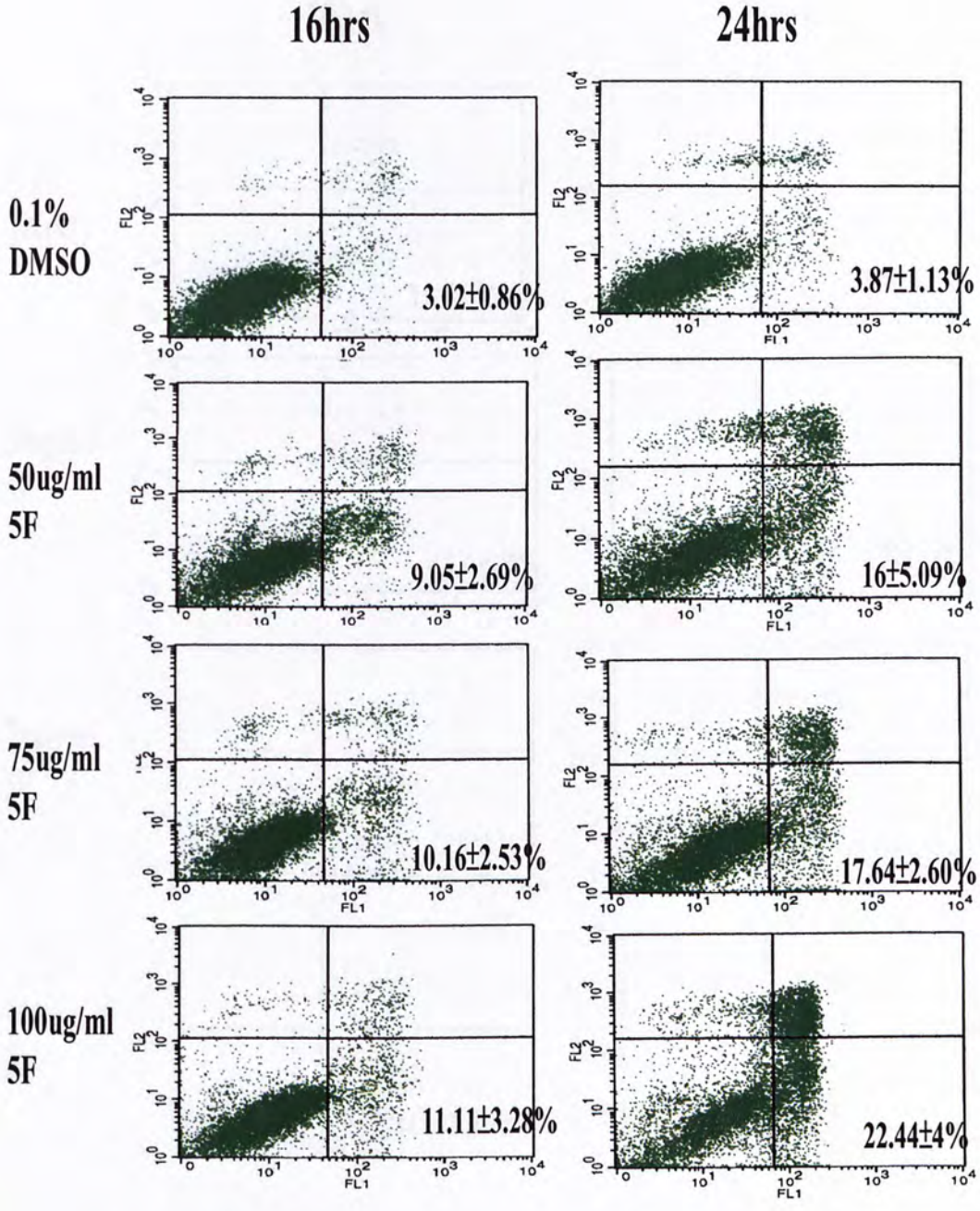


Figure 3.
5F induced apoptosis of UMSCC11A cells in dose-dependent manner. UMSCC11A cells were exposed to 5F (0, 50, 75 and 100 μg/ml) for 16 or 24hrs, and then subjected to Annexin V flow cytometry analysis to determine apoptosis.

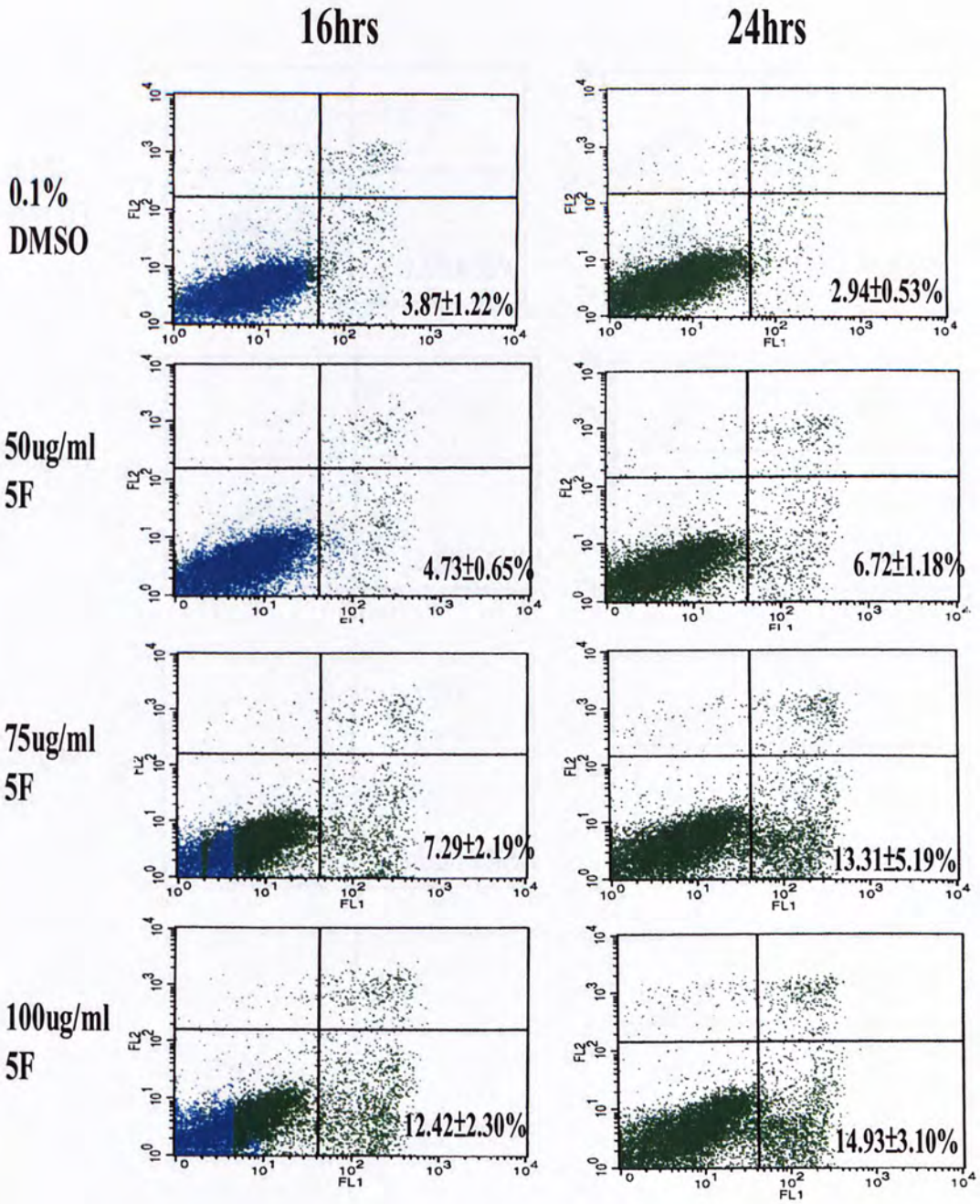


Figure 4.
5F induced apoptosis of UMSCC12 cells in dose-dependent manner. UMSCC12 cells were exposed to 5F (0, 50, 75 and 100 μg/ml) for 16 or 24hrs, and then subjected to Annexin V flow cytometry analysis to determine apoptosis.

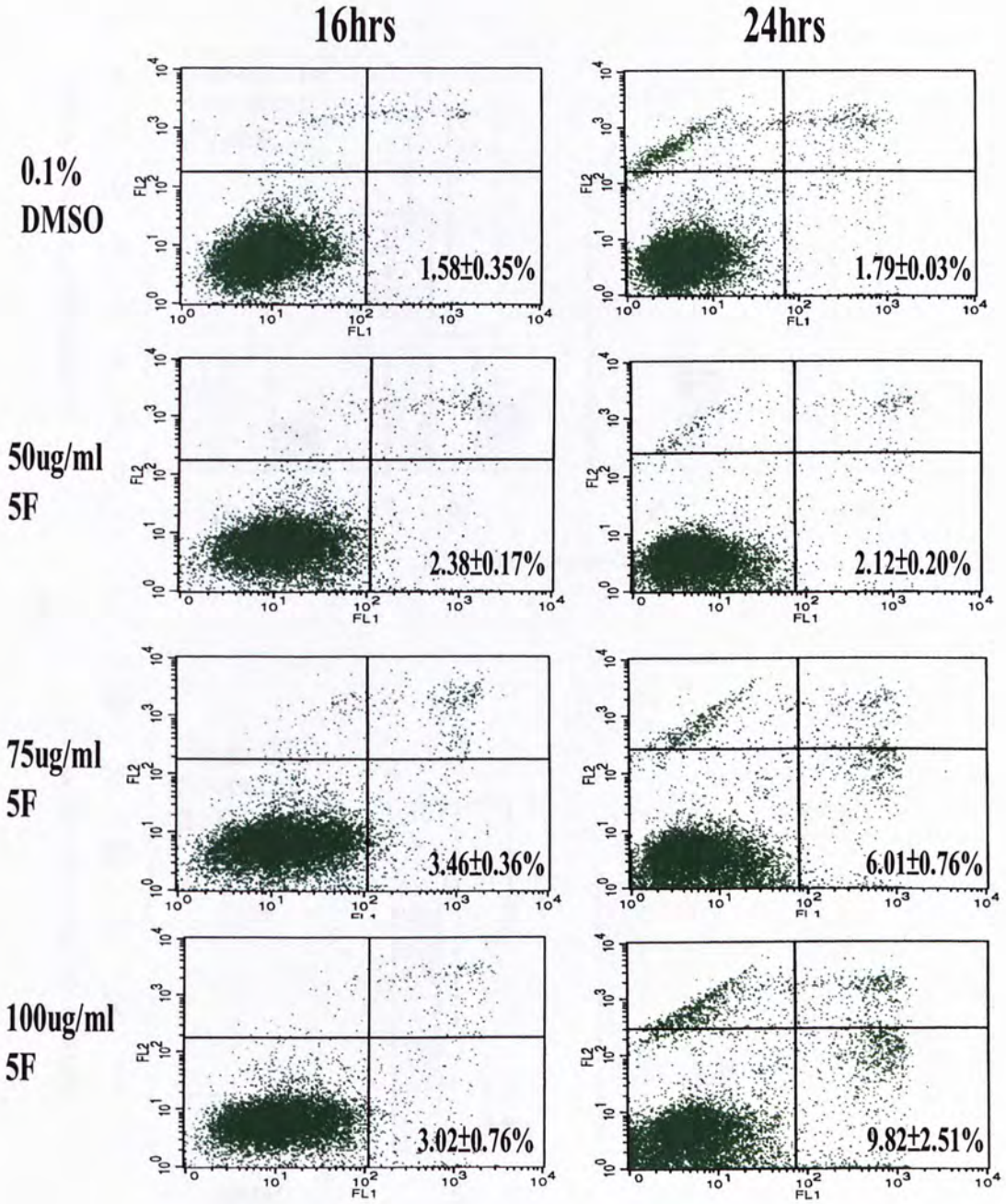


Figure 5.
5F induced apoptosis of HEp-2 cells in dose-dependent manner. HEp-2 cells were exposed to 5F (0, 50, 75 and 100 μg/ml) for 16 or 24hrs, and then subjected to Annexin V flow cytometry analysis to determine apoptosis.

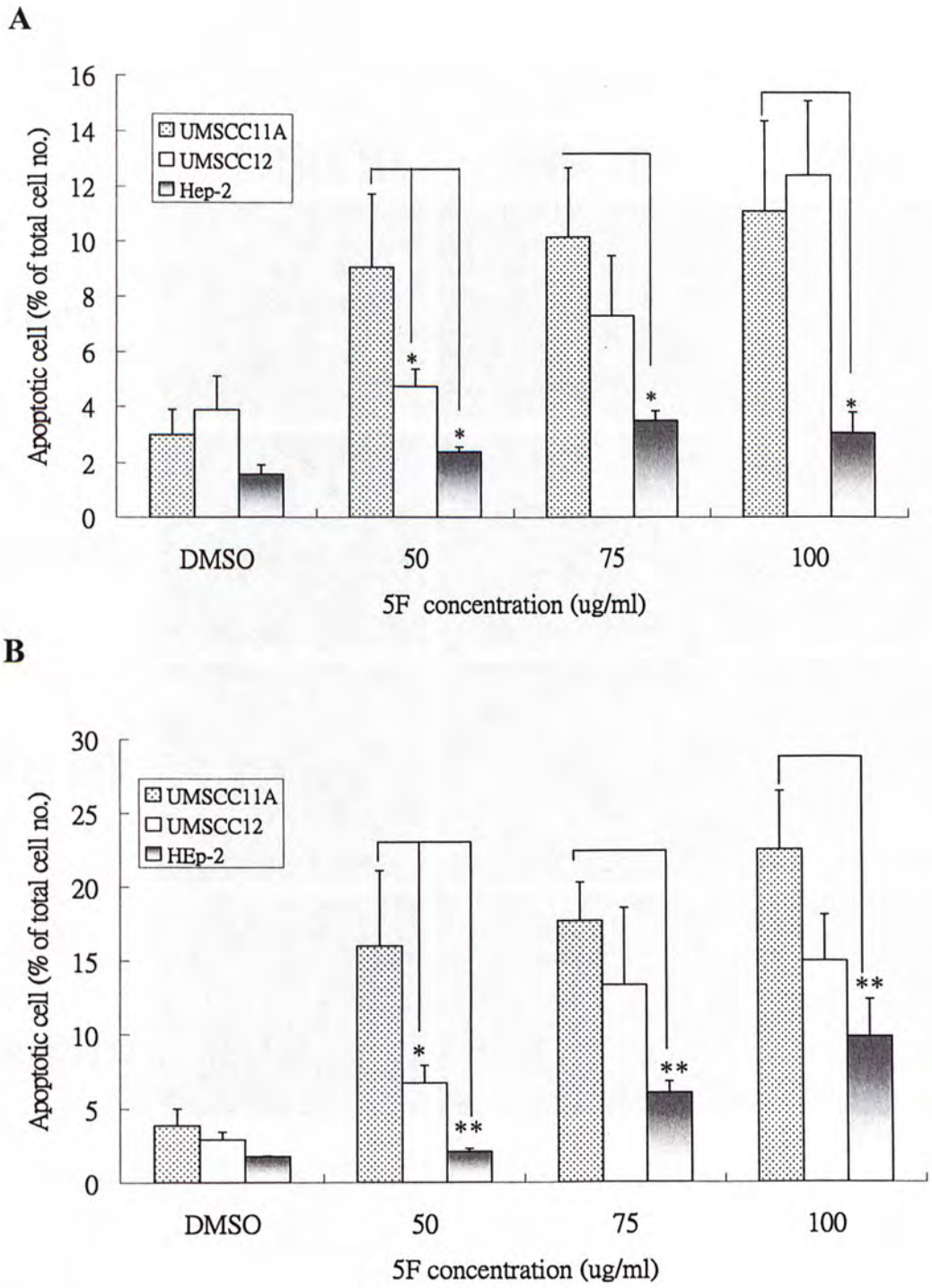


Figure 6.
Comparison of 5F-induced apoptosis in cell line UMSCC11A, UMSCC12 and HEp-2. After exposure to different doses of 5F (0, 50, 75 and 100 μg/ml) for 16hrs (A) or 24hrs (B), apoptosis was induced at different rates and magnitudes in the three cell lines (* $P < 0.05$, ** $P < 0.01$).

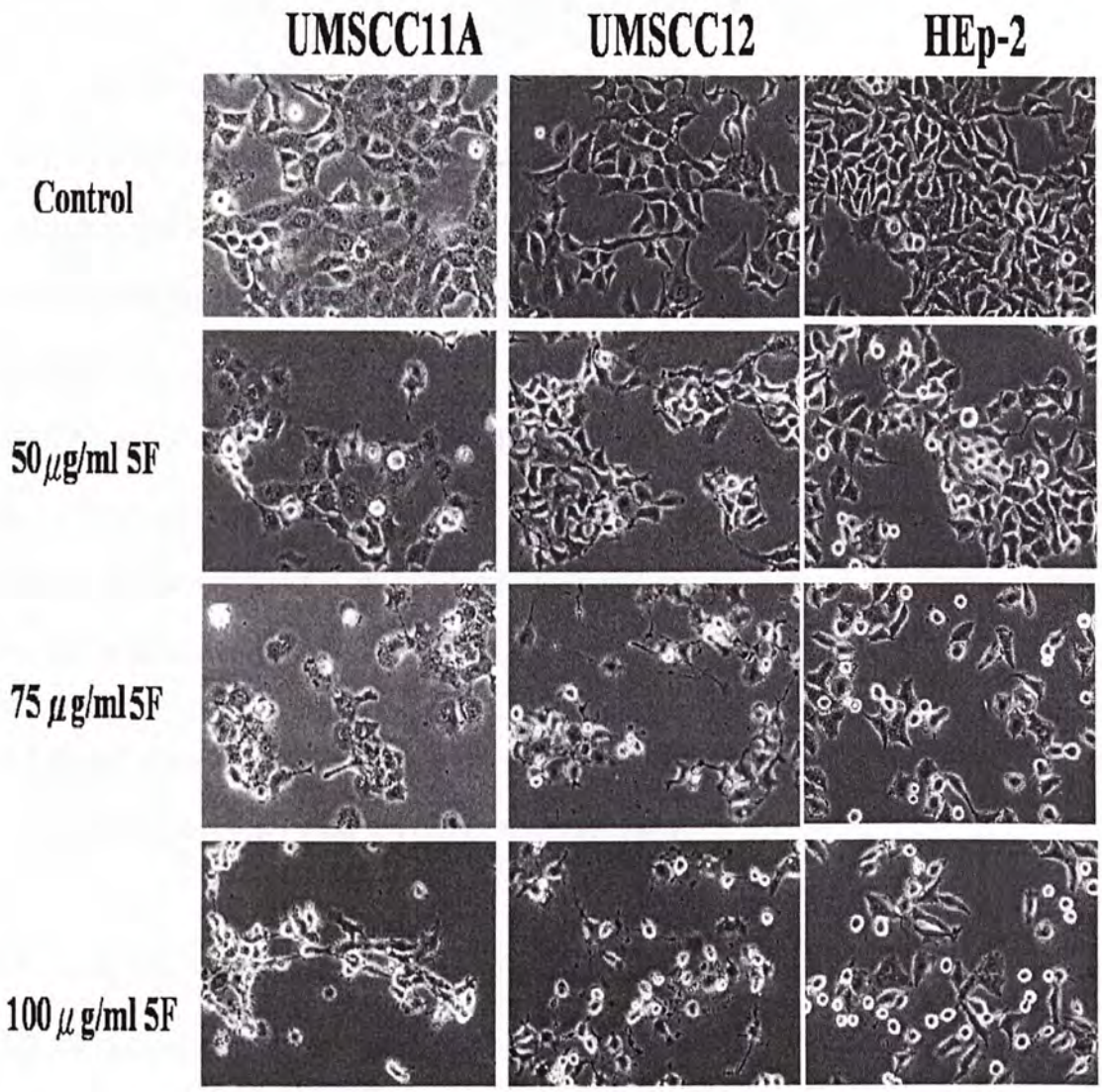


Figure 7.
5F induced cell morphology changes in UMSCC11A, UMSCC12 and HEp-2 cell lines. The cells were incubated with different concentrations of 5F (0.1% DMSO, 50, 75 and 100μg/ml) for 24hrs. Cell morphology was observed by a Nikon OPTIPHOT-2 fluorescence microscope at 200X magnification (Nikon, Tokyo, Japan)

3.5 Cleavage of poly (ADP-ribose) polymerase (PARP) and pro-caspase-3 induced by 5F in UMSCC11A, UMSCC12 and HEp-2 cell lines

To further investigate 5F-induced apoptosis in laryngeal cancer cells, Western Blotting was used to detect the cleavage of pro-caspase-3 and PARP, which are the hallmarks of apoptosis. On apoptosis, pro-caspase-3 is activated in order to cleave PARP, facilitating cellular disassembly. Cleavage of pro-caspase-3 and PARP appeared at 12 hours in UMSCC11A (Figure 8). In UMSCC12 and HEp-2 the activated caspase-3 and cleaved PARP were also detected at 24 hours. This indicates that 5F induced apoptosis as early as 12 hours in UMSCC11A, 24 hours in UMSCC12 and HEp-2. Densitometry analysis (Figure 9) showed that the relative level of cleaved caspase-3 and PARP increased with the 5F incubation time and peaked at 36 hours.

3.6 Down-regulation of TNF-α-induced NF-κB subunit p65 and p50 nuclear translocations in UMSCC11A, UMSCC12 and HEp-2 by 5F

The apoptosis inducing effect of 5F is known to be associated with the inhibition of NF-κB signaling in human colon cancers (Chen, Liang 2004) and the activity of NF-κB has been reported to be increased in laryngeal cancer tissue (Du, Chen 2003; Pan et al. 2005; Kourelis et al. 2007). However, whether 5F in laryngeal cancer acts on NF-κB is unknown. As NF-κB nuclear translocation is a crucial step for its signaling pathway, we analyzed the nuclear levels of NF-κB subunits p65 and p50 in 5F-treated UMSCC11A, UMSCC12 and HEp-2. TNF-α was used to stimulate the cellular translocation of p65/p50. Western Blotting for Lamin B and β-tubulin were used as a reference indicator

of equal loading of the nucleus and cytoplasm protein fraction. The levels of Lamin B and β-tubulin were almost undetectable in the cytoplasm and nucleus indicating that there was no contamination in the fractions. In the absence of the stimulus, the basal levels of nuclear p65/p50 were reduced to 0.2 fold in the presence of 50μg/ml 5F in all laryngeal cancer cells (Figure 10). On stimulation by 20ng/ml TNF-α, the levels of nuclear p65 and p50 greatly enhanced in the nucleus but their levels in the cytoplasm were reduced, indicating the nuclear translocation of the two proteins. In UMSCC11A, 1.5 and 3 folds increase of nucleus p65 and p50 occurred respectively after TNF-α activation. In UMSCC12, the level of nuclear p65 and p50 was drastically increased by 3.2 and 4.5 folds respectively. In HEp-2, 6 and 26 folds of nuclear p65 and p50 increments were observed. When cells were co-treated with TNF-α and different dosages of 5F, the p65 and p50 nuclear translocation was reduced by 5F in a dose-dependent manner. Interestingly, the reduced effect of p65 and p50 translocation by 5F in HEp-2 was weaker than in UMSCC11A and UMSCC12.

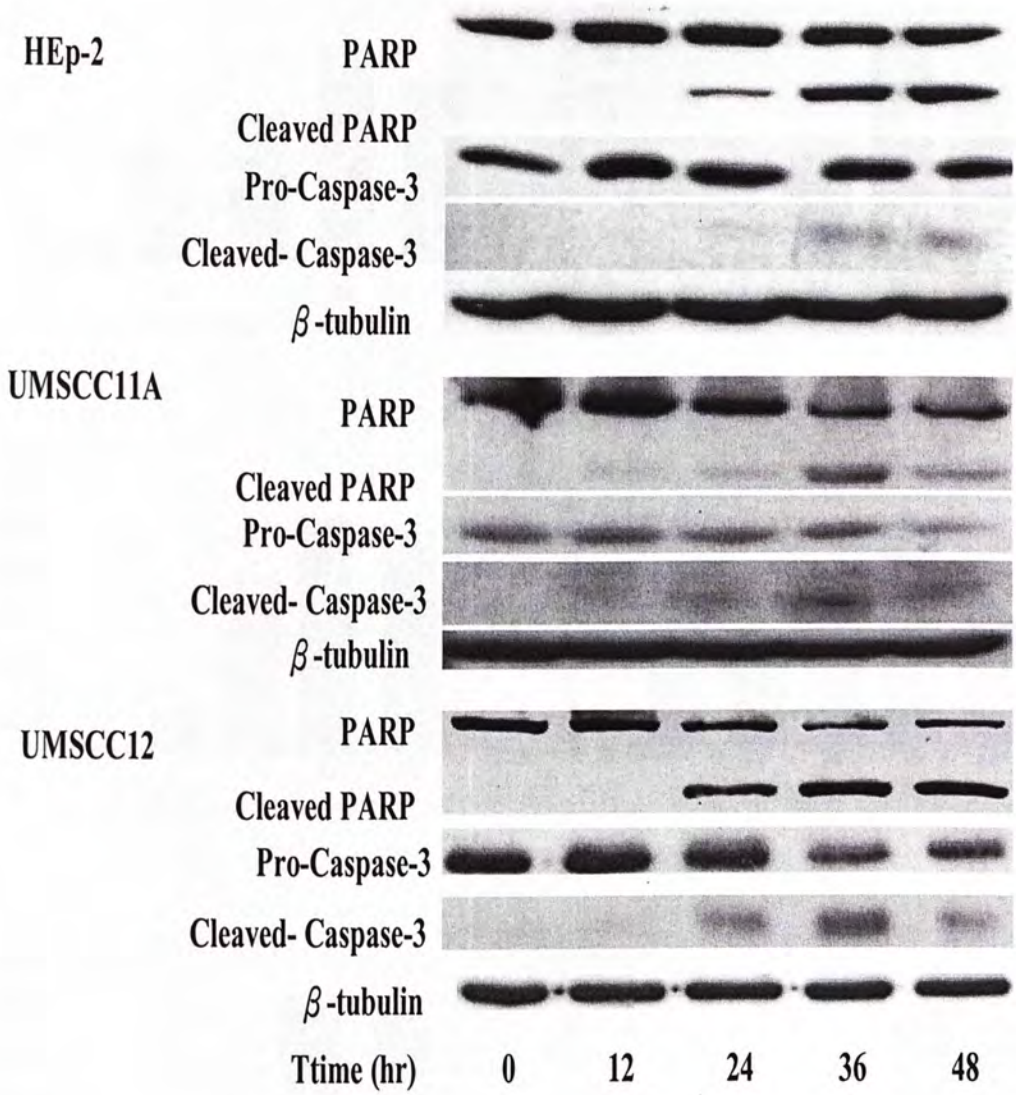


Figure 8.
5F induced cleavage of pro-caspase-3 and poly (ADP-ribose) polymerase in UMSCC11A, UMSCC12 and HEp-2 cell lines. UMSCC11A and UMSCC12 were treated with 50μg/ml 5F and HEp-2 with 75μg/ml 5F for different periods (0, 12, 24, 36 and 48 hours). Both attached and floating cells were collected and lysed to detect the apoptosis hallmarks, caspase-3 and poly (ADP-ribose) polymerase, by Western Blotting.

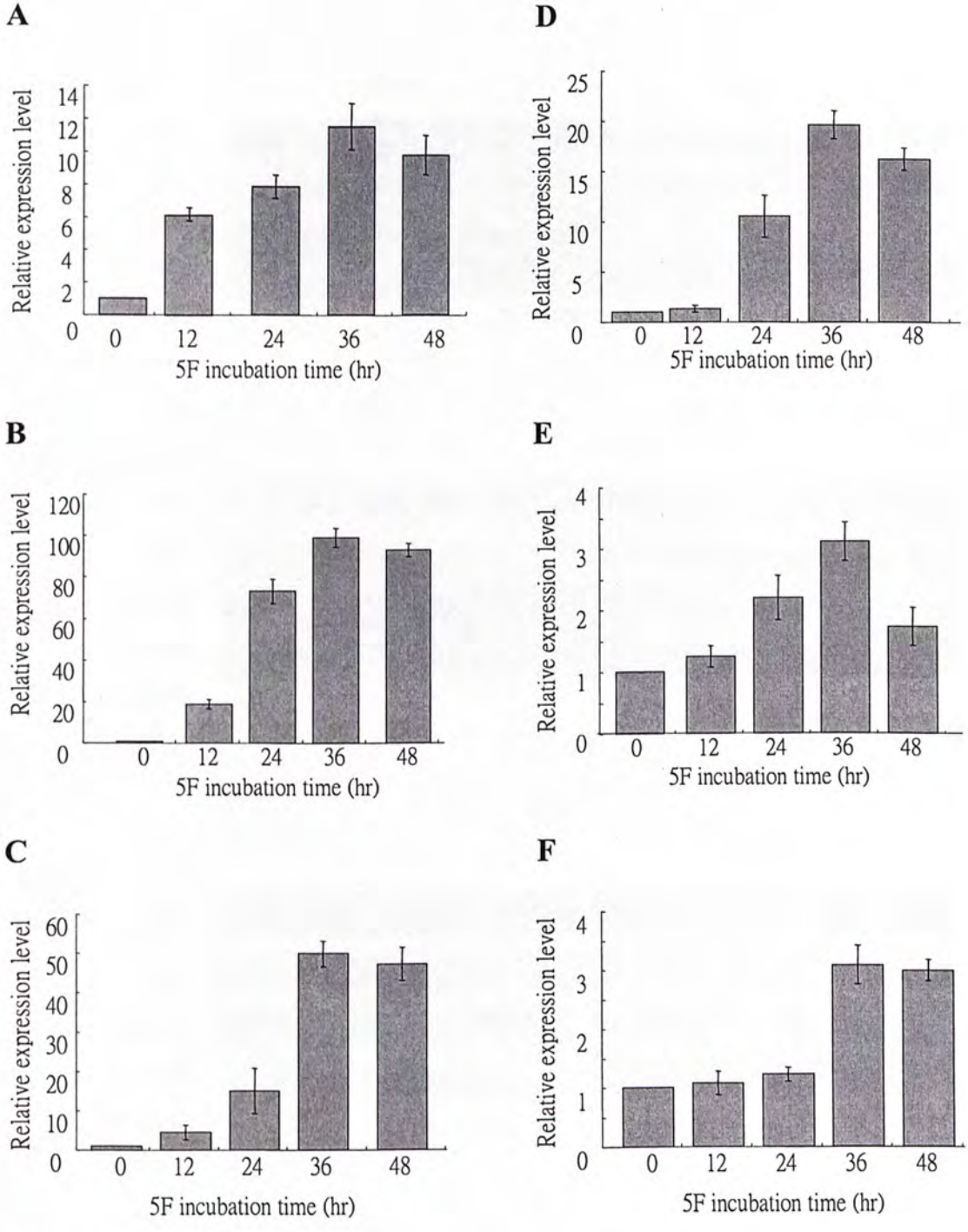
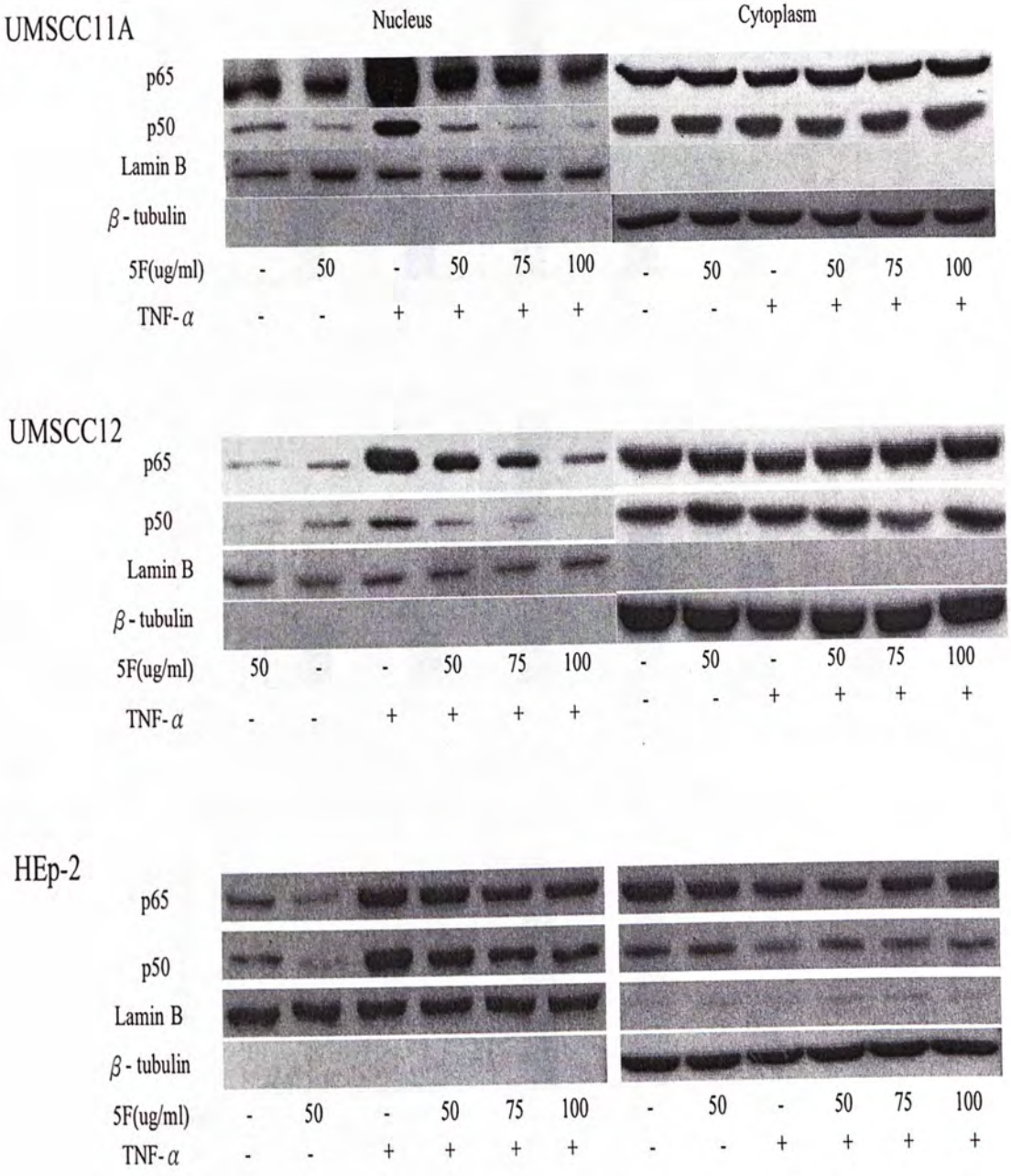


Figure 9.
Densitometry analysis of cleaved caspase-3 and PARP in UMSCC11A, UMSCC12 and HEp-2 cell lines. Table A, B and C respectively represent cleavage of PARP in UMSCC11A, UMSCC12 and HEp-2. Table D, E and F represent cleavage of pro-caspase-3 in UMSCC11A, UMSCC12 and HEp-2 respectively.


UMSCC11A
Nucleus
Cytoplasm
p65
p50
Lamin B
β - tubulin
5F(ug/ml) - 50 - 50 75 100 - 50 - 50 75 100
TNF-α - - + + + + - - + + + +
UMSCC12
p65
p50
Lamin B
β - tubulin
5F(ug/ml) 50 - - 50 75 100 - 50 - 50 75 100
TNF-α - - + + + + - - + + + +
HEp-2
p65
p50
Lamin B
β - tubulin
5F(ug/ml) - 50 - 50 75 100 - 50 - 50 75 100
TNF-α - - + + + + - - + + + +

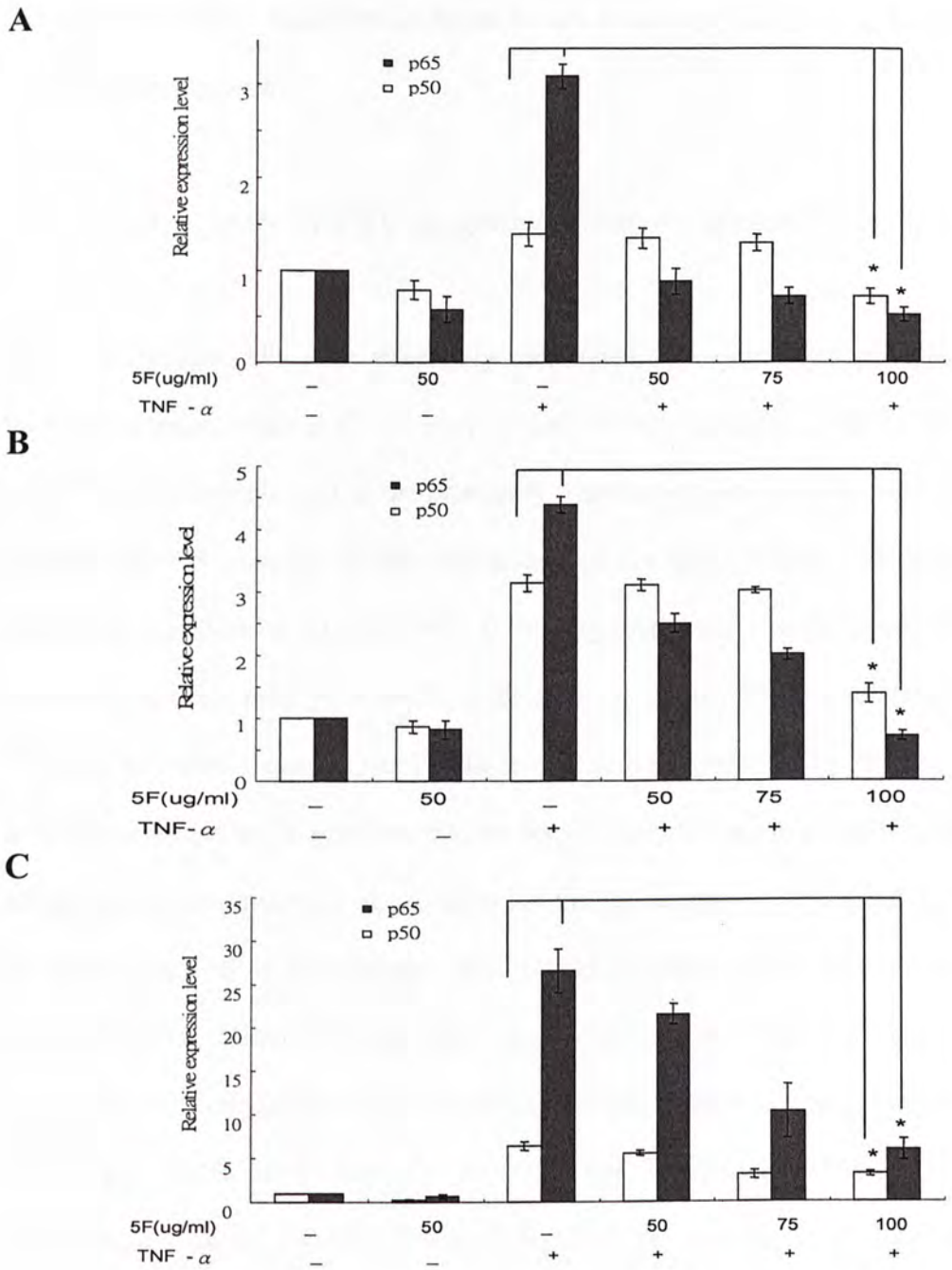


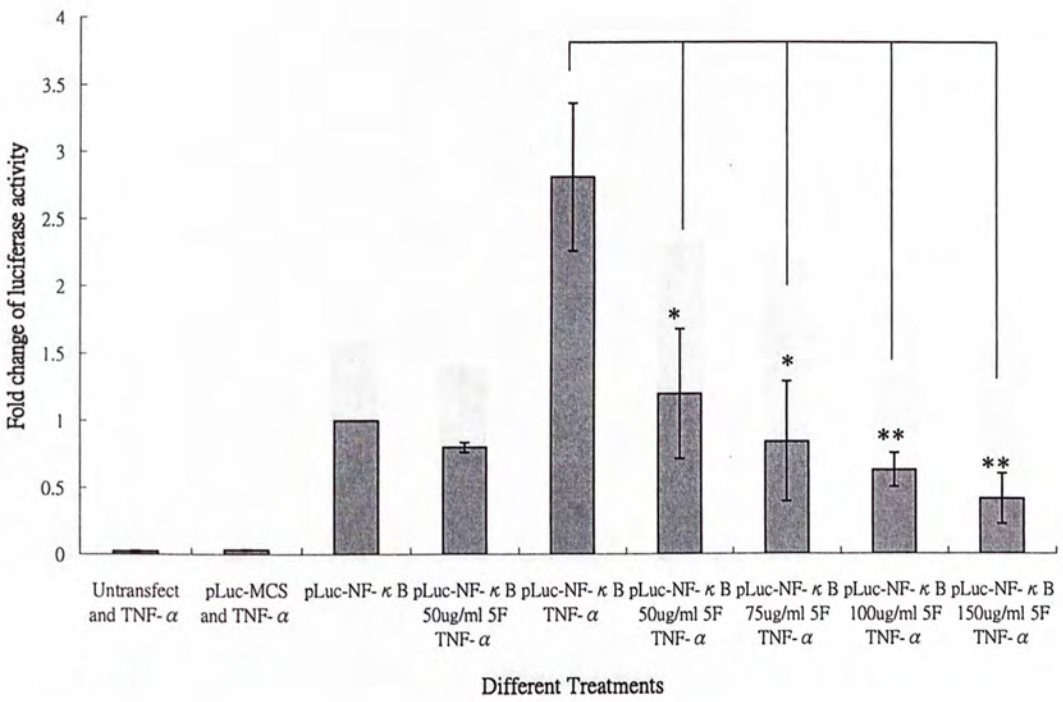
Figure 10.5F inhibits the TNF-α-mediated translocation of NF-κB subunits p65 and p50. UMSCC11A, UMSCC12 and HEp-2 cells were pre-treated with different dosages of 5F (50, 75 and 100 μg/ml) in serum free medium for 2 hours. Cells were then stimulated with 20ng/ml TNF-α for 20 min. The nuclear and cytoplasm proteins were isolated to detect p65 and p50 proteins. The relative expression level of the nuclear p65 and p50 proteins in UMSCC11A (A), UMSCC12 (B) and HEp-2 (C) are represented by the bar chart. (* $P < 0.05$)

3.7 Dose-dependent inhibition of 5F on NF-κB transcriptional activity measured by luciferase assay

The effect of 5F on the NF-κB transcriptional activity was examined by luciferase assay.

Laryngeal cancer cells were transiently transfected with pLuc-MCS plasmid (control vector containing minimal TATA box) or pLuc-NF-κB plasmid (containing five repeats of NF-κB response element in the promoter). The signal generated from the pLuc-MCS transfection was regarded as the background of the assay (Figure 11). Without any treatment, transfection of pLuc-NF-κB in laryngeal cancer cells generated higher luciferase activity than pLuc-MCS, indicating the basal NF-κB activation status in different laryngeal cancer cells. The luciferase activity generated by the basal NF-κB activity was regarded as a reference value for the other treatment. In the presence of 5F alone, the luciferase activity due to basal NF-κB activation was suppressed 0.2 fold. On stimulation by TNF-α, the luciferase activity was increased 3 folds, 16 folds and 2 folds in UMSCC11A, UMSCC12 and HEp-2 respectively. With 50μg/ml 5F pre-treatment, the TNF-α induced luciferase activity was significantly inhibited 3 folds in UMSCC11A ($P<0.05$), 7 folds in UMSCC12 ($P<0.05$), and 1.4 fold in HEp-2. Our results demonstrate that 5F inhibits TNF-α-induced NF-κB transcriptional activation in a dose-dependent manner.

A


Fold change of luciferase activity
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
**
**
Untransfect and TNF- α
pLuc-MCS and TNF- α
pLuc-NF- κ B
pLuc-NF- κ B 50ug/ml 5F TNF- α
pLuc-NF- κ B TNF- α
pLuc-NF- κ B 50ug/ml 5F TNF- α
pLuc-NF- κ B 75ug/ml 5F TNF- α
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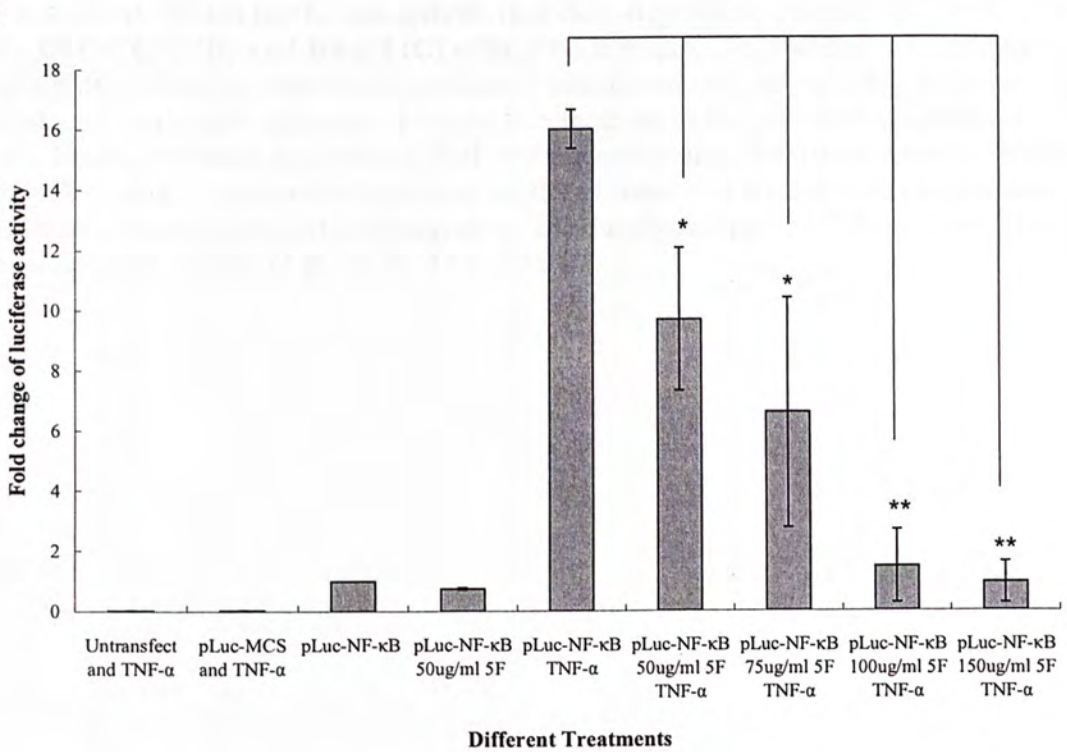
pLuc-NF- κ B 150ug/ml 5F TNF- α
Different Treatments

B


Fold change of luciferase activity
18
16
14
12
10
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6
4
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Untransfect and TNF-α
pLuc-MCS and TNF-α
pLuc-NF-κB
pLuc-NF-κB 50ug/ml 5F
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pLuc-NF-κB 150ug/ml 5F TNF-α
Different Treatments

C

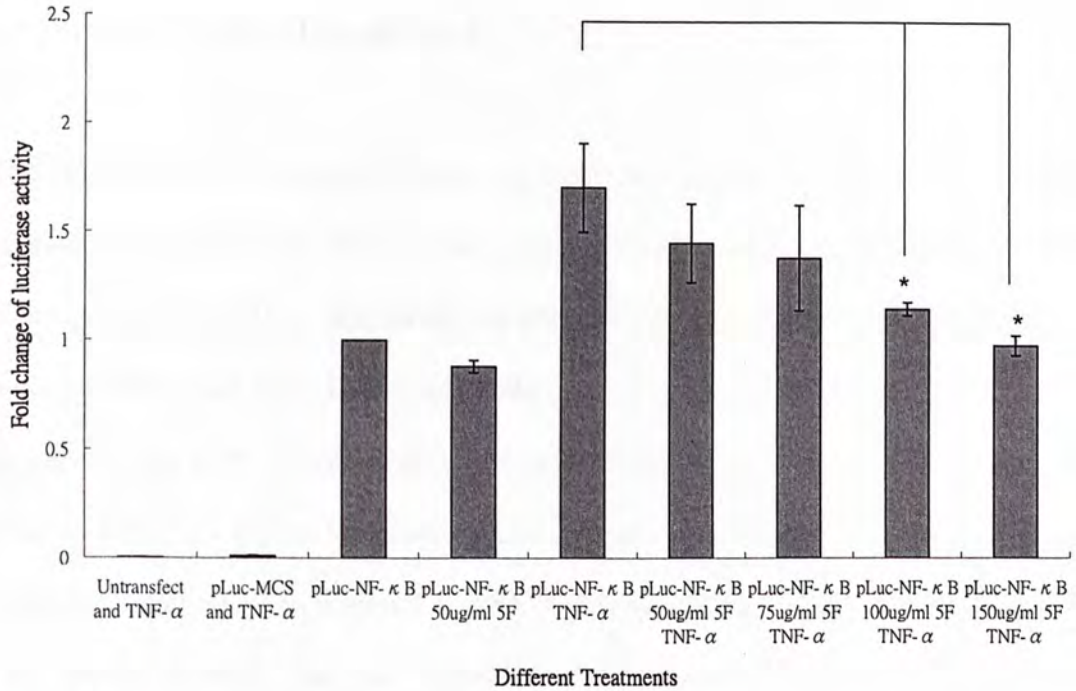


Figure 11.
5F inhibited NF-κB luciferase activity in a dose-dependent manner in UMSCC11A (A), UMSCC12 (B) and HEp-2 (C) cells. NF-κB responsive elements were cloned into a pLucMCS plasmid, which was transiently transfected into the cells for 24 hours. The transfected cells were cultured in serum free medium in the presence or absence of 5F for 2 hours, followed by 20ng/ml TNF-α for another 6hrs. On stimulation by TNF-α, p65/p50 bound to responsive elements on the plasmid and triggered the expression of luciferase which generated luminescence. The luciferase activity reflected the NF-κB transcriptional activity (* $P < 0.05$, ** $P < 0.01$).

3.8 Partial inhibition of TNF-α induced IκBα degradation by 5F in UMSCC11A but not in UMSCC12 and HEp-2

The induction of p65 and p50 nuclear translocation by TNF-α is known to involve the degradation of IκBα. We thus investigated whether 5F suppressed the degradation of IκBα induced by TNF-α. We found that 50μg/ml 5F did not alter the basal expression level of IκBα and that TNF-α stimulated the degradation of IκBα (Figure 12) in laryngeal caner cells. When UMSCC11A cells were pre-treated with 5F for 2 hours, the level of IκBα was higher than that with TNF-α only. In contrast, the levels of IκBα in UMSCC12 and HEp2 in response to TNF-α were not altered by pre-treatment with 5F. Our finding showed that 5F suppressed TNF-α-induced NF-κB translocation by retardation of IκBα degradation in UMSCC11A, but not in UMSCC12 or Hep-2. This suggests that 5F can affect TNF-α-induced NF-κB translocation in laryngeal cancer cells through different mechanisms.

3.9 Cell proliferation inhibition and apoptosis induction by Bay (11-7082) in laryngeal cancer cells

Our results demonstrated 5F induced apoptosis and inhibited the nuclear translocation and transcription activity of NF-κB. However, whether the suppressed NF-κB activity in laryngeal cancer cells could result in apoptosis is unknown. To address this point, we used specific NF-κB inhibitor Bay (11-7082) to verify the effect of NF-κB inhibition on apoptosis induction in laryngeal cancers.

We found that Bay (11-7082) slowed down the cell proliferation rate of laryngeal cancer cells in a dose-dependent manner (Figure 13) after 24 and 48 hours of treatment. This confirmed that the inhibition of the NF-κB pathway by Bay (11-7082) led to the retardation of cell proliferation. In the Annexin V assay, 26.05% (± 3%) UMSCC11A cells, 2% (±0.4%) UMSCC12 and 2% (± 0.8%) HEp-2 underwent apoptosis after 24 hours of treatment with 10μM Bay (11-7082) (Figure 14). Our results from Annexin V assay confirmed that inhibition of NF-κB activity resulted in laryngeal cancer cell apoptosis. However, the inhibition of the NF-κB activation induced a high level of apoptosis only in UMSCC11A cells, but not in UMSCC12 or HEp-2 cells.

3.10 Differential basal nuclear translocation of p65 and p50 in laryngeal cancer cell lines

UMSCC11A showed a higher sensitivity to 5F or Bay (11-7082) treatments than UMSCC12 and HEp-2. We believe that this could be due to the different basal levels of nuclear p65 and p50 in various laryngeal cancer cell lines. By Western blotting, both nuclear p65 and p50 were found to be diminished by the action of 5F. Even though the nuclear levels of p65 and p50 were decreased by about 20% in all laryngeal cancer cells tested after the 2-hour 5F treatment, the basal levels of nuclear p65 in the UMSCC11A cell line was higher than that in UMSCC12 and HEp-2 while the levels of p50 were similar in all three cell lines (Figure3.15). This difference indicates that the inhibition of NF-κB plays a more important role in apoptosis induction in UMSCC11A than in UMSCC12 and HEp-2. Consistent with this idea, we have shown that 5F induces a higher degree of apoptosis and that Bay (11-7082) only induces apoptosis in

UMSCC11A but not in UMSCC12 or HEp-2.

3.11 5F regulation of NF-κB target gene expression

NF-κB activation exerts its carcinogenic effects via transcriptional activation of its target genes. Cylin D1 is over-expressed in a variety of cancers and is responsible for the progress of the cell cycle from the G1 to the S phase (Polsky and Cordon-Cardo 2003). Similarly, COX-2 is also over-expressed in cancer cells and mediates cell proliferation (Chun and Surh 2004). The role of EGFR is important in cell proliferation and DNA synthesis (Nishi et al. 2003). Survivin, which belongs to the inhibitor of apoptosis protein (IAP) family, is a negative regulator of apoptosis by inhibiting caspase activation (Zucchini et al. 2008). Bax is a pro-apoptotic regulator and responsible for the permeabilization of the outer mitochondrial membrane (van Delft and Huang 2006). We thus examined the effects of 5F on the expression of these NF-κB-regulated gene products in laryngeal cancer cells by Western blotting. We successfully identified a number of NF-κB target genes that were altered by 5F in a time-dependent manner (Figure 16). These include proliferation-related genes (COX-2, EGFR and cyclin D1), anti-apoptotic gene (survivin), and pro-apoptotic gene (Bax).

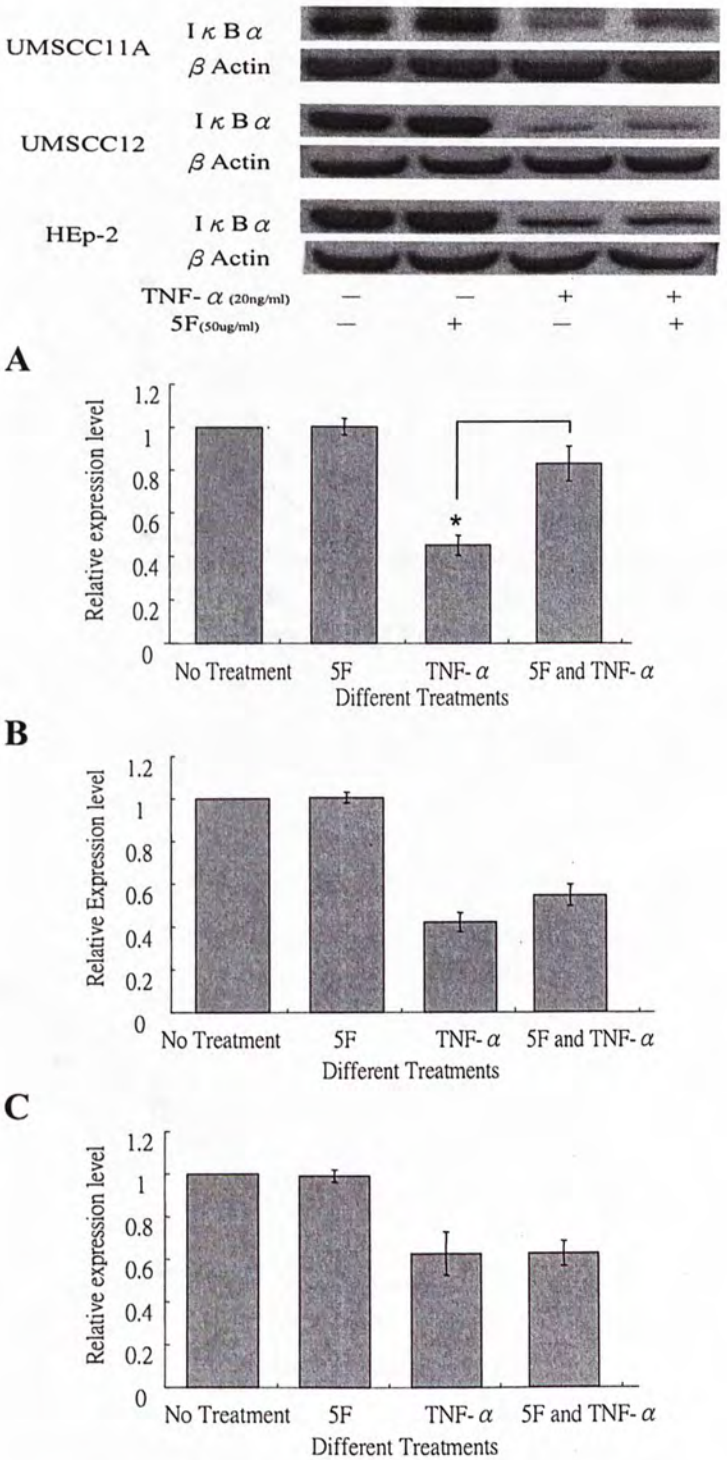


Figure 12.

5F inhibited TNF-α-induced IκBα degradation in UMSCC11A but not in UMSCC12 or HEp-2 cells. UMSCC11A (A), UMSCC12 (B) and HEp-2 (C) cells were pre-treated with 50μg/ml 5F in serum free medium for 2 hours, and then stimulated by 20ng/ml TNF-α for 15min. The protein was isolated and subjected to Western blotting analysis for IκBα ($P < 0.05$).

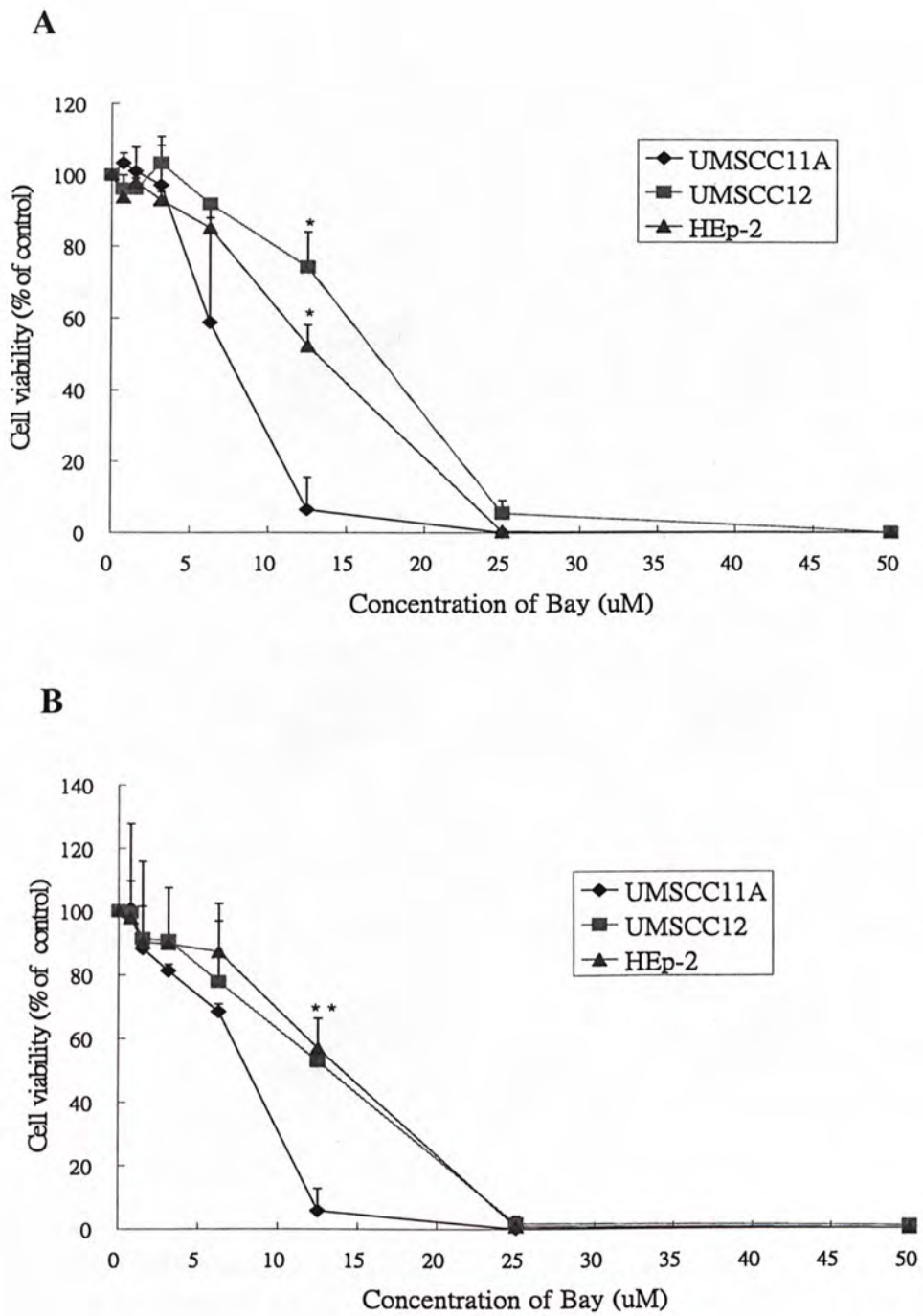


Figure 13.

Anti-proliferation effect of Bay (11-7082) in UMSCC11A, UMSCC12 and HEp-2 cell lines. UMSCC11A, UMSCC12 and HEp-2 cells were treated with different dosages of Bay (0.1% DMSO, 1.5625, 3.125, 6.25, 12.5, 25 and 50uM) for 24 (A) or 48 (B) hours. Cell proliferation was determined by MTT assay. The MTT assay was performed as described in materials and methods. Values shown represent the mean of three separate experiments with each experiment performed in triplicate. ($P < 0.05$)

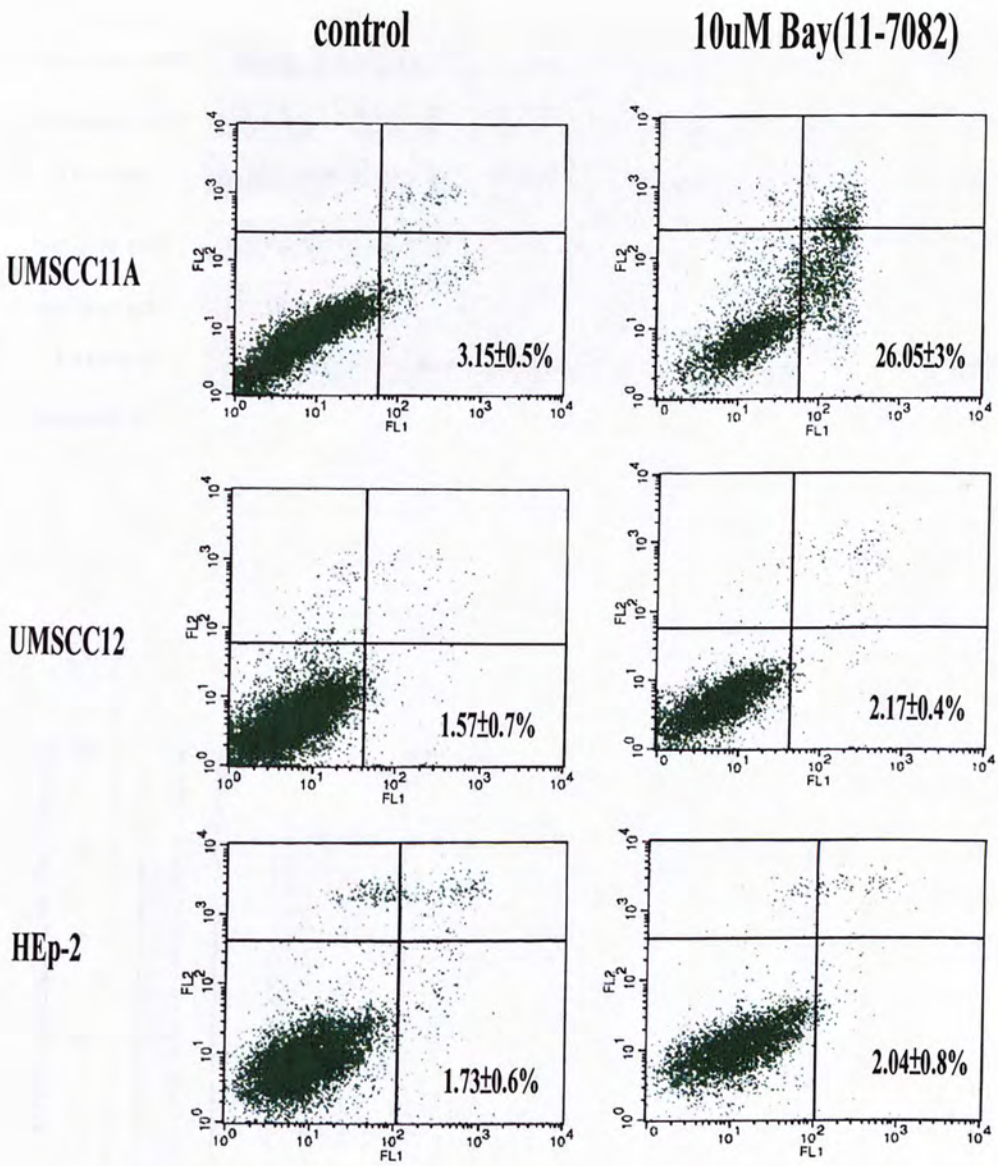
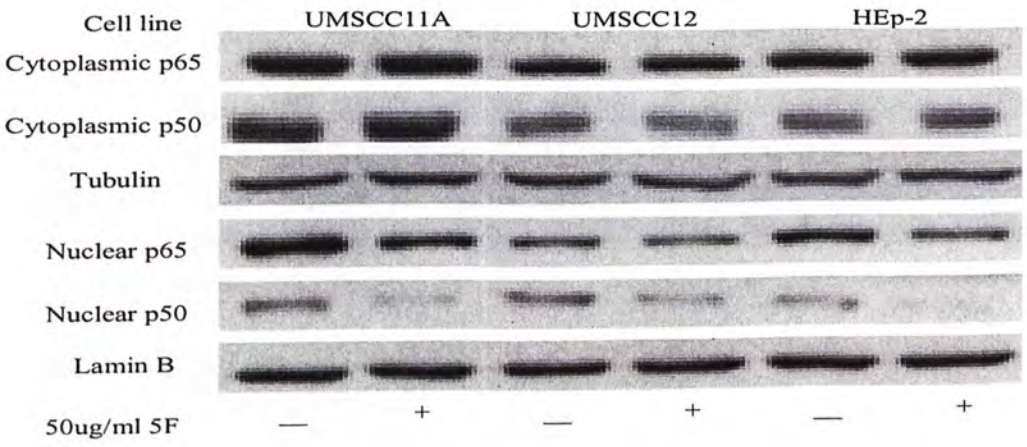


Figure 14
Bay (11-7082) induced apoptosis in UMSCC11A. UMSCC11A, UMSCC12 and HEp-2 were exposed to 10uM Bay for 24 hours, and subjected to Annexin V flow cytometry analysis to determine apoptosis. Values shown represent the mean of three separate experiments.



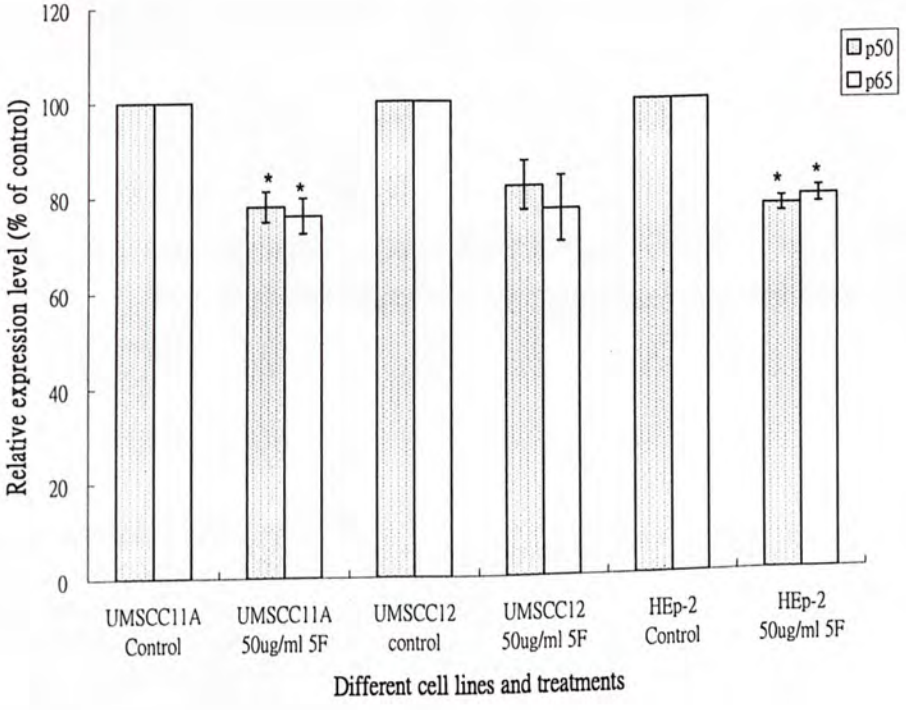


Figure 15.
5F decreased the nuclear level of p65/p50 proteins in UMSCC11A, UMSCC12 and HEp-2. Cells were exposed to 50μg/ml 5F for 2 hours. After incubation, different cellular fractions were isolated and subjected to Western blot analysis. The relative expression levels of nuclear p65/p50 proteins were determined by densitometry on the Western blot signals (* $P < 0.05$).

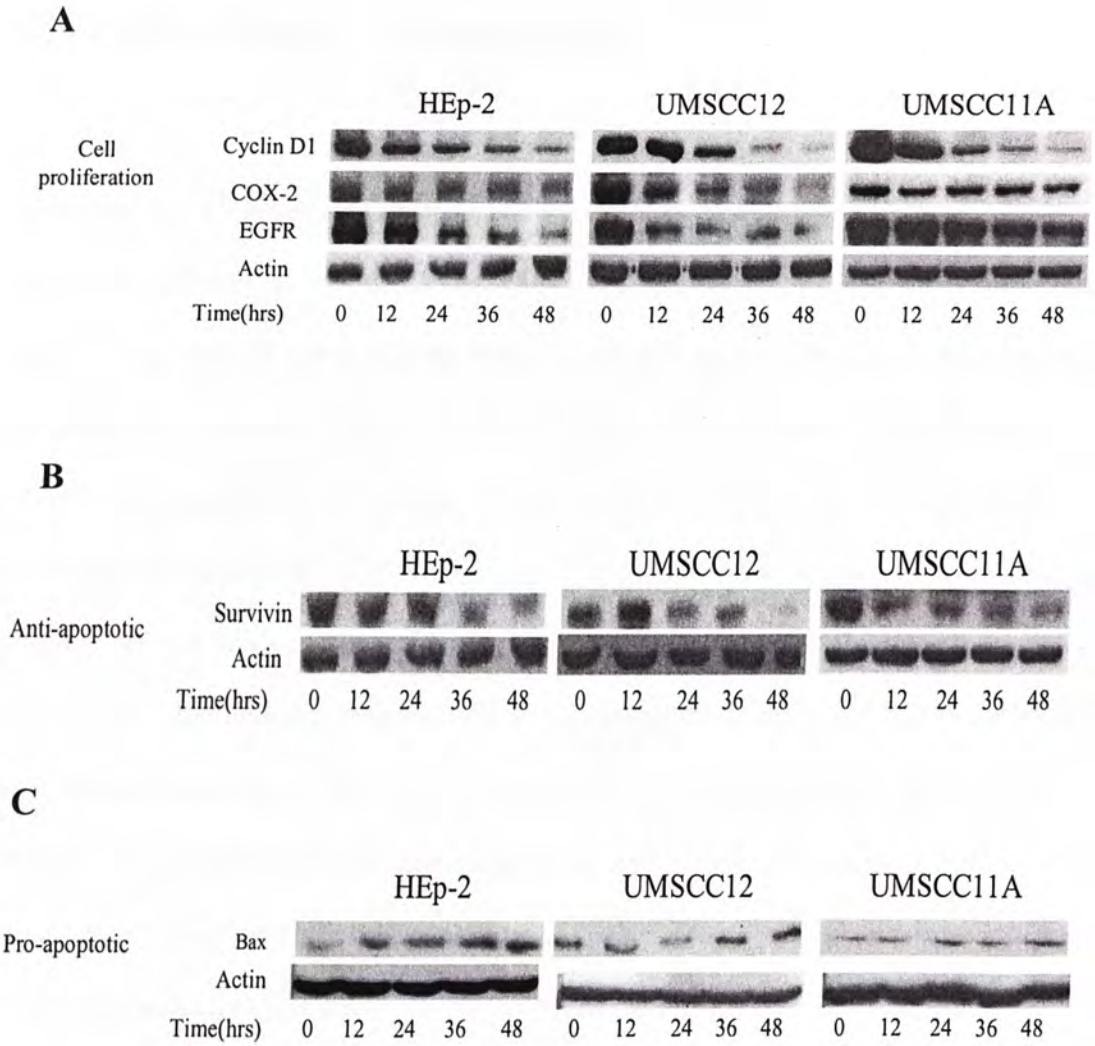


Figure 16.
5F down-regulated the expression level of NF-κB target genes. UMSCC11A and UMSCC12 were exposed to 50μg/ml 5F and HEp-2 was exposed to 75μg/ml 5F for different periods (0, 12, 24, 36 and 48hrs). Identifiable NF-κB target genes were detected by Western blotting (A) Expression of cell proliferation regulators: cyclinD1, COX-2 and EGFR; (B) Expression of anti-apoptotic regulator: survivin and (C) Expression of pro-apoptotic regulator: Bax.

Chapter Four Discussion

Herbal extract 5F is known to inhibit cell growth in several types of tumor. However, its effect on human laryngeal cancer cells has not been studied yet. Our results demonstrated that 5F can effectively retard the proliferation of laryngeal cancer cells in a does-dependent manner. And this inhibitory effect on proliferation was attributed to the 5F-induced apoptosis. Furthermore, we demonstrated that 5F significantly suppressed the transcription activity of NF-κB via the blockage of NF-κB nuclear translocation. Specific NF-κB inhibitor, Bay (11-7082), also gave similar effects on laryngeal cancer cell growth inhibition and apoptosis induction. In response to the 5F-suppressed NF-κB activity, the expression levels of NF-κB target gene products were also down-regulated. Herein, we showed that the down-regulation of NF-κB transcription activity was involved in 5F-induced apoptosis in laryngeal cancer cells. Our study provides evidence for 5F as an effective therapeutic agent for targeted therapy in laryngeal cancer cells.

As apoptosis inactivation is common in tumors and contributes to carcinogenesis, apoptosis induction is thus one of the most obvious strategies for cancer therapy. It has been shown that the NF-κB signaling pathway and its downstream gene products are critical regulators of the apoptotic responses (Karin and Lin 2002). Constitutive activation of NF-κB is found to be a common feature in various human cancer cells including laryngeal cancer cells (Du, Chen 2003; Pan, Tao 2005; Pacifico and Leonardi 2006). Nevertheless, the functional role of NF-κB in laryngeal cancer remains to be investigated. Here, through direct inhibition of NF-κB nuclear translocation by 5F, we

demonstrated that constitutive NF-κB activation is required for laryngeal cancer cell growth and apoptosis inhibition.

5F induced apoptosis has been shown in human colon cells via down-regulation of the NF-κB activity (Chen, Liang 2004). Our results are consistent with previous findings in which 5F suppressed the basal and TNF-α-induced NF-κB activity in laryngeal cancer cells (Figure 11). In the absence of stimulus, NF-κB is sequestered into the cytoplasm by binding with IκBα in the classical pathway. Therefore, the basal NF-κB activity observed in our luciferase assays was due to the constitutive basal activation of NF-κB in laryngeal cancer cells. This was further confirmed by the presence of nuclear p65 and p50 protein in the absence of stimulus (Figure 15). Upon stimulation with TNF-α, IκBα is phosphorylated by the IKK complex and then degraded subsequently. Since nuclear translocation is the last step of the NF-κB activation cascade preceding NF-κB-dependent transcription, blocking of NF-κB nuclear translocation is a major target for NF-κB inhibitors. IκBα is the commonest targets of numerous NF-κB inhibitors acting on NF-κB or its up-stream signaling pathway. The inhibitory action of these inhibitors on IκBα degradation often results in an increase in the level of IκBα. Bay (11-7082) is an irreversible inhibitor of IκBα phosphorylation and the subsequent proteasomal degradation. Thereby, Bay (11-7082) sequesters NF-κB in its inactive state in the cytoplasm and prevents the activation of NF-κB downstream signaling. We demonstrated that 5F inhibited the activation of NF-κB by partially suppressing the degradation of IκBα in UMSCC11A. The inactivation of NF-κB by 5F was independent of IκBα degradation in UMSCC12 and HEp-2 (Figure 12). Rather 5F might inhibit NF-κB activation in UMSCC12 and HEp-2 by masking its nuclear translocation signal

or blocking the nuclear transporter of NF-κB. In fact, there are evidences showing that same NF-κB inhibitors can act on different points along the NF-κB pathway in different cells (Karin et al. 2004). Sulphasalazine, a non-steroid anti-inflammatory drug, suppresses NF-κB activation via blocking the phosphorylation and degradation of IκBα in response to stimuli. However, 5-amino-salicylic acid, an oral administrated derivative of sulphasalazine, was shown to block NF-κB activation through inhibiting IKK-α and IKK-β kinase activity (Wahl et al. 1998; Yan and Polk 1999). Since the permeability or uptake of 5F may be cell line specific, its inhibition mechanism in UMSCC12 and HEp-2 needs to be further investigated.

Our experiments showed that 5F induced apoptosis and inhibited the NF-κB activity in laryngeal cancer cells. However, this did not mean that NF-κB inhibition in laryngeal cancer cells induced apoptosis. To address this, we studied the effects of Bay (11-7082) on the NF-κB activation and the apoptosis induction in laryngeal cancer. Our MTT assays showed that the cell proliferation rate was retarded in the presence of 10μM of Bay (11-7082). Annexin V assay showed that apoptosis was induced by Bay (11-7082) (10μM) in UMSCC11A. Given that Bay (11-7082) specifically inhibits the NF-κB activity, these results indicated that the effects of Bay (11-7082) in laryngeal cancer cells were attributed to the inhibition of NF-κB function. Therefore, we conclude that NF-κB is involved in the 5F-induced apoptosis in laryngeal cancer cells. Since NF-κB is a multi-functional regulator for cell proliferation and apoptosis, our findings further support that the perturbation of NF-κB function is a potentially useful therapeutic strategy for laryngeal cancer treatments.

We also found it interesting that Bay (11-7082) only significantly induced apoptosis in UMSCC11A but not in UMSCC12 and HEp-2. Indeed, in other cancers, mere inhibition of NF-κB is insufficient for inducing apoptosis (Karin 2006). We believe that the differential levels of basal NF-κB activation in various laryngeal cancer cells may contribute to the response differences to 5F in UMSCC11A, UMSCC12, and HEp-2. UMSCC11A exhibited a relatively higher constitutive NF-κB activity than that in HEp-2 and UMSCC12 (Figure 15). This indicated that NF-κB played a more important role in cell survival in UMSCC11A than that in UMSCC12 and HEp-2. Similar situation has been reported by Angela A et al (Van Waes et al. 2005). Bortezomib, a novel proteasome inhibitor, suppresses a panel of HNSCC cell lines (including UMSCC11A, -11B) via inhibiting NF-κB activation with different sensitivity. However, the cell lines with low constitutive NF-κB activity were more resistant to bortezomib while those with high activity such as UMSCC11A and UMSCC 11B are more sensitive to bortezomib. On the other hand, it is noticeable that the higher constitutive NF-κB activity in UMSCC11A might be due to the relatively lower expression level of IκBα (Figure 12). By comparing the finding from the treatment of 5F and Bay (11-7082), we suggested that 5F induced apoptosis in laryngeal cancer cells involving other mechanisms in addition to NF-κB signaling pathway.

Other than low constitutive NF-κB activity, the presence of HPV18 may also contribute to the resistance to 5F for inducing apoptosis in laryngeal cancers. We found that HEp-2 was more resistant to 5F than that in UMSCC12. Hep2 is a HPV18 positive cell line. Two cooperative oncoproteins E6 and E7 are encoded in the genome of HPV 18. E6 is responsible for cancer progression whereas the function of E7 is primarily for inducing

transformation (Song et al. 2000; Scheffner and Whitaker 2003). The small interfering RNAs (siRNAs) targeting E6 and E7 inhibits the growth of cervical cancer cells (Fujii et al. 2006). Suppression of E6 and E7 by antisense RNAs also significantly induces apoptosis in HeLa cells (Sima et al. 2007). Therefore, E6 and E7 are essential to the growth of human HPV-positive laryngeal cancers. Furthermore, the complex cooperation between E6 and E7 is essential for malignant transformation (von Knebel Doeberitz et al. 1988; zur Hausen 1994). Yamato et al reported that E6-specific suppression alone induced more apoptosis than the E6 and E7 co-suppression in HPV 18 positive cervical cancer cells (Yamato et al. 2006). It is known that E7 inactivates cell cycle regulators like pRb in order to up-regulate genes for G1/S transition and DNA synthesis. Moreover, E7 also causes the up-regulation of pro-apoptotic genes, such as c-Myc, Bak and p53 (Askew et al. 1991). However, in HEp-2, the E6 can rescue the cells from cell death by binding with these pro-apoptotic proteins for subsequent degradation (Gross-Mesilaty et al. 1998; Thomas and Banks 1998). Therefore, it is conceivable that without E6 co-expression, E7 can induce cell death by activating pro-apoptotic genes. On the other hand, suppression of E7 may induce cell growth arrest rather than cell death. Here, we demonstrated that 5F suppressed the mRNA and protein expressions of E7 whereas the expression of E6 was not altered. However, the reduced E7 expression by 5F did not enhance apoptosis significantly in HEp-2, when comparing with UMSCC12. This might be due to the fact that the 5F-induced E7 reduction was too subtle and the unaltered E6 level still exerted a significant anti-apoptotic effect. To address the apoptotic roles of E6 and E7 in laryngeal cancer cells, we suggest to evaluate the effects on apoptosis after silenting both genes in HEp-2, or introducing them into HPV-negative laryngeal cancer cells like UMSCC11A and UMSCC12 in future.

NF-κB regulates both cell proliferation and apoptosis via its different target genes. After addressing the inhibitory effect of 5F on the NF-κB nuclear translocation, we then showed that 5F down-regulated a number of NF-κB known target genes in laryngeal cancer cells such as proliferation-related (COX-2, EGFR and cyclin D1), anti-apoptotic (survivin), as well as pro-apoptotic genes (Bax). Expression of COX-2, EGFR and survivin has been shown to have value in predicting prognosis in laryngeal squamous cell carcinoma (Pan, Tao 2005; Marioni *et al.* 2006; Wei *et al.* 2008). Increasing expression of survivin accompanied with laryngeal papilloma malignancy (Poetker et al. 2002). The cell cycle and differentiation regulator EGFR is over-expressed in laryngeal papillomas and cultured papilloma cells (Johnston et al. 1999). Constitutive activation of NF-κB has also been shown in laryngeal papillomas and HPV-positive laryngeal cancers (Vancurova *et al.* 2002; Du, Chen 2003). Up-regulation both of COX-2 and NF-κB has been shown to be a feature of laryngeal carcinogenesis (Pan et al. 2005; Kourelis et al. 2007). Given that NF-κB up-regulates COX-2, which then activates EGFR signaling (Sarkar and Li 2008), down-regulation of COX-2 and inactivation of EGFR via NF-κB inhibition can be used synergistically for killing cancer cells. Also, inhibition of NF-κB can lead to the suppression of survivin and EGFR and then prevents the malignant transformation of laryngeal papillomas. Therefore, our observation of the COX2, survivin and EGFR down-regulation by 5F may be worthy for future development of laryngeal papilloma treatments.

NF-κB signaling pathways have various effects on laryngeal carcinogenesis. It becomes one of the most attractive targets for cancer prevention and therapy. Here, we have

demonstrated that an herbal extract 5F effectively inhibits the growth of laryngeal cancer cells via induction of apoptosis. Moreover, the suppression of NF-κB activity by 5F subsequently inhibits the expression of the NF-κB target genes responsible for cell proliferation and apoptosis. Our results implicate that 5F is a new potent anti-tumor agent for laryngeal cancers by targeting its NF-κB signaling pathway.

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Appendix

Appendix 1 Map of pLuc- NF-κB plasmid: