國立臺灣大學醫學院臨床牙醫學研究所

碩士論文

Graduate Institute of Clinical Dentistry

College of Medicine

National Taiwan University

Master Thesis

# 細胞凋亡抑制蛋白 1 於口腔鱗狀細胞癌與口腔癌前病變之表現

# Expression of cellular inhibitor of apoptosis protein 1 in oral squamous cell carcinomas and precancerous lesions

張家銓

Chia-Chuan Chang

指導教授：江俊斌 教授

Advisor: Professor Chun-Ping Chiang

中華民國 97年 6月

June, 2008

# 謝　　誌

能夠完成這篇論文，獲得碩士學位，要感謝的人實在是太多了。

首先，我要感謝中山醫學大學的黃裕峰醫師，雖然他是在我大學四年級時才回國，開始接受教職，但他生動有趣的教學方法，有條不紊的依年齡症狀等資料，一一條列出來的診斷方式，並且在臨床上實際地教學，把臨床上呈現的病症，參照以課本上的內容，這樣的教學方式，開啟我對口腔疾病的興趣，是帶領我了解口腔疾病的導師。

大我一屆的蔡東潔學姊，也是我研究所的學姊，在我還是實習醫師的階段，準備研究所的甄試時，將她當時甄試的經驗，可能的考試方式，以及一些重點整理的筆記，毫不保留的告訴我。她熱心地幫助我，讓我能夠順利地推甄上研究所，我很感謝她。在博士班的余權航學長，是我進入研究所之後，最常接觸也最常麻煩的學長。從一開始的認識環境，從病理科，門診，圖書館，餐廳的位置，一一帶我去實際走過；到研究所上課的課程選擇，報告的順序，寫論文的方式，如何找需要的資料，如何整理找到的資料，到後來做實驗時的過程，甚至小到影印機的問題，幾乎無所不包，受學長的幫助太多，也麻煩他太多了。

而一開始病理科的訓練，麻煩最多的就是王逸平學長了。一開始接牙科檢體，看著切下來的下頷骨，看著切下來的頸部廓清，是王逸平學長，從如何，orientation，如何 ink，如何切，如何找 lymph node，到切片出來後的教學，都是勞煩王逸平學長。而在病理科期間，最要感謝的是在同一間辦公室的謝明書學長和周岳宏學長，以及另一間辦公室的李仁傑學長和王中傑學長，還有搞笑的林維洲學長。在面對大病的 case 時，他們教導我許多的知識，教導我如何取下需要的檢體，也教導我如何看切片，尤其是謝明書學長，是被我麻煩最多的學長，一些可能對於他們來說都是"秒殺"的切片，我一再地麻煩他，他還是不厭其煩得教導，真的很謝謝他。還有系辦的呂姐，學校的大小瑣事，都是由呂姐她所提醒，幫助的；還有我研二才回國的張玉芳學姊，雖然教我的時間不長，但如何做切片的診斷，臨床表現的鑑別，教導我許多，謝謝！

我的指導教授，江俊斌醫師，他是一個溫文儒雅的學者，沒有一般印象中，所謂教授的嚴厲。從上課，論文題目的選定，過程中的討論，老師都幫助我許多。或許我不認真，常常將一些雜亂無章，不能稱之為成果的結果拿給老師看，隔天，老師就會幫我整理出重點，並且告知我可能還需要加強的地方。對於這篇論文，老師幫助我最多，讓我學會如何做研究，如何擬定題目，如何尋找資料，如何整理所得，進而完成這篇論文。在老師身上學到的，不只是研究的方法，論文的寫

作，更甚的是待人的態度。在研究室，我看到老師對於我，只要我有問題去找他，他一定先放下手邊的事，幫我處理我的問題；在門診，我看到老師對於患者的視病猶親，將患者的病痛相當重視，重視他們的無助與感受。這些在在都影響了我，影響我處事的態度，也讓我知道，身為一位醫師應有的責任。

我要感謝我的女朋友素惠，我和她從大學時期即已交往，至今已有七年的歲月。我是一個性情有些急燥的人，對於某些事情不如意，可能便會影響我的心情，而素惠她便是最直接承受我脾氣的人。從大學期間每一次的期考，考試前對於未念完的心情；每一學期一定會有的實驗作品，無法做出理想狀態的急燥態度等，都是她包容我，體諒我，讓我能夠安心向前。實習期間初次面對醫療上的挫折，是素惠她每天安慰我，給予我鼓勵；邊忙於實習遇到的困難，還要忙於推甄的許多瑣事，是素惠她和我討論，給予我意見，對於審查資料的製作幫我查資料，幫我修改，幫我檢查，是她幫助我許多。除此之外，在我需要人陪，孤單寂寞，覺得徬徨無助時，或許別人看不出來，但都是她陪我走過，給我鼓勵，給我安慰，她是給予我莫大動力與支持的人。

最後，我要感謝我的父母，張榮顯先生和張吳麗華女士。你們是最開明的父母，從小對我們的教育，不會以強迫的方式使我們學習，他們總是尊重我，愛護我，苦口婆心的教育我。還記得，上大學時，因我是家中的么兒，送我到學校之後，依依不捨地離開，甚至落下淚來的心情，我便能體會他們是多麼愛我的。你們是世界上最好的父母，雖然並非富裕家庭，但靠著父母的一雙手，讓我能夠無後顧之憂地繼續求學，甚至只要是對於我將來有好處的，你們都願意付出，只希望我有更好的將來。你們是影響我最大父母，你們對於我的重視，從小到大的各種活動，參加考試，畢業典禮，你們幾乎都是全程參與，從不缺席，讓我的成長過程，知道有你們的支持，知道有你們當作我的後盾，我會更加努力，謝謝！

# 目　　錄

## Tables

## Figures



# 中文摘要

**背景**: 細胞凋亡抑制蛋白 1 (cIAP-1)的過度表現，已經在不同的人類癌症中被發現，且和腫瘤的大小，淋巴結的轉移，腫瘤分期，復發和預後等有關。

**方法**: 在本研究中，我們利用免疫組織化學染色法，探討 cIAP-1 蛋白質在 73 例口腔鱗狀細胞癌（OSCC），76 例口腔上皮變異（OED；23 例輕度，34 例中度，19 例重度上皮變異），31 例正常口腔黏膜（NOM）之表現。計算 cIAP-1 在 OSCC、OED、和 NOM 細胞質的染色強度(staining intensity, SI)和染色指標(labeling indices, LIs，定義為在所有細胞中陽性染色細胞的百分比)並比較組間差異。利用統計分析 OSCCs 細胞質 LIs 和臨床參數或存活率間的關連性。

**結果**: 結果顯示平均細胞質 cIAP-1 LIs 從 NOM (23±22%)， 經 OED (50±25%) 至 OSCC 樣本 (73±17%)，呈統計上有意義增加 (NOM v.s. OED or OSCC, *P*=0.000; OED v.s. OSCC, *P*=0.000)。平均細胞質 cIAP-1 LIs 和 OSCCs 和局部淋巴轉移 (*P*=0.000)，和較高的臨床分期(*P*=0.045)有明顯相關。

**結論**: 我們的結果顯示，cIAP-1 廣泛地表現在正常的、變異的與惡性的口腔上皮細胞之細胞質中。而 cIAP-1 在細胞質的表現從 NOM 至 OED 至 OSCC 有顯著的增加。量測 OSCC 樣本細胞質 cIAP-1 的表現，也許可預測口腔癌的進程、復發和預後。

關鍵字: 細胞凋亡抑制蛋白 1，口腔癌，口腔癌前病變

# Abstract

*Background:* Overexpression of cellular inhibitor of apoptosis protein 1 (cIAP-1) has been demonstrated in a variety of human cancers and found to be associated with the lymph node metastasis, clinical stage, recurrence, or prognosis of these cancers.

*Methods:* In this study, we examined the expression of cIAP-1 protein in 73 specimens of oral squamous cell carcinoma (OSCC), 76 specimens of oral epithelial dysplasia (OED), and 31 specimens of normal oral mucosa (NOM) by immunohistochemistry. The cytoplasmic cIAP-1 labeling indices (LIs) in OSCC, OED, and NOM samples were calculated and compared between groups. The correlation between the cytoplasmic cIAP-1 LI in OSCCs and clinicopathological parameters or survival of OSCC patients was analyzed statistically.

*Results:* The mean cytoplasmic cIAP-1 LIs increased significantly from NOM (23 ± 22%) through OED (50 ± 25%) to OSCC samples (73 ± 17%) ($P = 0.000$). A significant correlation was found between the higher mean cytoplasmic cIAP-1 LIs and OSCCs with positive lymph node metastasis ($P = 0.000$) or more advanced clinical stages ($P$=0.045).

*Conclusion*: Our results suggest that the increased expression of cIAP-1 is an early event in oral carcinogenesis and the cIAP-1 may be a biomarker for OSCCs. Measuring the amount of cytoplasmic cIAP-1 expression in OSCC samples may predict the oral cancer progression in Taiwan.



**Key words**: cellular inhibitor of apoptosis protein 1, oral cancer, oral precancer

# Introduction

Oral squamous cell carcinoma (OSCC) is the fifth most common malignancy and is a major cause of cancer morbidity and mortality worldwide. Globally about 500,000 new oral and pharyngeal cancers are diagnosed annually, and three quarters of these are from the developing world (Nagpal and Das 2003). In Taiwan, oral cancers ranks as the sixth most prevalent cancer in both sexes and account for the fourth most common cancer in males (Cancer registry annual report, ROC, 2007). Ko et al. (1995) found that the incidence of oral cancer in Taiwan is 123-fold higher in patients who smoke, drink alcohol, and chew AQs than in abstainers.

Tissue homeostasis relies on the balance between cell proliferation and cell death. Apoptosis, or programmed cell death, is a complex and highly regulated process essential for proper development and functioning of multicellular organisms and for the removal of damaged or infected cells. Defects in the regulation of apoptosis are involved in the pathogenesis of a variety of human diseases, including cancer and autoimmune diseases. The caspase family of cysteine proteases plays an essential role in orchestrating the orderly breakdown of cells that characterizes apoptosis. The organism must tightly regulate the caspase cascade, which starts with the activation of upstream or initiator caspases and leads to the activation of downstream or effector caspases.

Inhibitor of apoptosis (IAP) proteins negatively regulate the apoptotic program by interfering with caspase activity. IAP proteins are characterized by the presence of at least one 70-amino acid N-terminal domain called baculovirus IAP repeat (BIR). To date, eight human IAPs have been identified: cIAP-1, cIAP-2, NAIP, survivin, XIAP, Bruce, ILP-2, and livin.

cIAP-1 is a member of IAP family protein. It is composed of three BIR domains, a

caspase recruitment (CARD) domain, and a carboxy-terminal RING domain. Previous studies have demonstrated the overexpression of cIAP-1 in lung (Ferreira et al. 2001, Dai et al. 2003), cervical (Imoto et al. 2002), prostate (McEleny et al. 2002, Krajewska et al. 2003), esophageal (Imoto et al. 2001), head and neck (Tanimoto et al. 2005), and colorectal carcinomas (Krajewska et al. 2005). In these studies, overexpression of cIAP-1 is correlated with positive lymph node metastasis, more advanced clinical stage, and poor prognosis in head and neck carcinomas (Tanimoto et al. 2005) and with poorer overall and local recurrence-free survival in cervical SCCs (Imoto et al. 2002).

The messenger RNA (mRNA) encoding for cIAP-1 has been detected in different types of normal human tissues. In the context of normal tissues, these IAPs have been involved in the regulation of the immune system, the response to cell damage, and the cell survival and differentiation. Cellular levels and activity of IAPs are controlled by several mechanisms, including transcriptional regulation, via nuclear factor-κB, and posttranslational modification, via proteasome degradation. The activity of IAPs can be further modulated by 2 IAP-binding proteins, Smac/Diablo and Omi/Htra2, which prevent their association with caspases.

Although cIAP-1 has been shown to correlate with progression of some cancers, the expression of cIAP-1 in OSCCs in Taiwan has not yet been studied. Furthermore, the correlation between the expression of cIAP-1 in OSCCs and the clinicopathological parameters or survival of OSCC patients was not known. Therefore, the main purposes of this study were to examine the expression of cIAP-1 in OSCCs and to assess its correlation with the clinicopathological parameters or survival of OSCC patients.

# Background

Oral cancer evolves in a series of distinct steps, each characterized by the sequential accumulation of additional genetic defects followed by clonal expansion. In neoplasms, cell proliferation is excessive and autonomous, uncoordinated with normal tissue with cell division going on despite DNA damage due to loss of cell cycle check-points (Nagpal and Das 2003). Although our previous studies have showed that overexpression of cyclin D1 (Kuo et al. 1999), cyclin A (Chen et al. 2003), p53 (Chiang et al. 1999), $p21^{WAF1}$ (Kuo et al. 2002), survivin (Lin et al. 2005), and hypoxia inducible factor (HIF)-1α (Lin et al. 2008) correlates with poorer prognosis in patients with AQ chewing and cigarette smoking-related OSCCs, lymph node metastasis is still the most significant factor determining the prognosis of oral cancer patients.

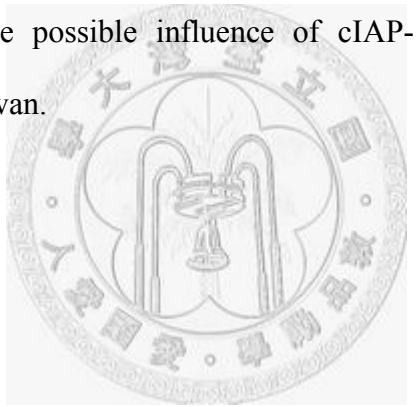
Life and death of cells must be balanced if tissue homeostasis is to be maintained. The main (though not the only) death mechanism by which mammalian cells maintain homeostasis is apoptosis. Dysregulation of apoptosis clearly contributes to the pathogenesis of various human diseases including cancer. Defects in the apoptotic pathway can eventually lead to expansion of a population of neoplastic cells and affect the intrinsic ability to respond to therapy.

Overexpression of cIAP-1 protein has been demonstrated in a variety of human carcinomas including lung (Ferreira et al. 2001, Dai et al. 2003), cervical (Imoto et al. 2002), prostate (McEleny et al. 2002, Krajewska et al. 2003), esophageal (Imoto et al. 2001), head and neck (Tanimoto et al. 2005), and colorectal carcinomas (Krajewska et al. 2005). Results from previous studies suggest that cIAP-1 may be a biomarker for human malignancies and may play an important role in human carcinogenesis.

In some of these carcinomas, the expression of cIAP-1 protein has been found to be significantly associated with the lymph node metastasis, clinical stage, and prognosis

of the cancer patients. However, the influence of cIAP-1 expression on the progression and prognosis of AQ chewing and tobacco smoking-related OSCCs in Taiwan has not yet been investigated.

In this study we investigated the expression of cIAP-1 in 73 specimens of OSCC, 76 specimens of oral epithelial dysplasia (OED, 19 severe, 34 moderate, and 23 mild OED cases), and 31 specimens of normal oral mucosa (NOM) by immunohistochemistry using antibodies to cIAP-1 protein. The cIAP-1 labeling indices (LIs) in OSCC, OED, and NOM samples were calculated and compared between groups. The correlation between the expression of cIAP-1 protein in OSCCs and the clinicopathological parameters or survival of the OSCC patients was analyzed statistically to evaluate the possible influence of cIAP-1 on the progression and prognosis of OSCCs in Taiwan.



# Purposes of this study

1. To examine the expression of cIAP-1 in specimens of NOM, OED, and OSCC
2. To compare the cIAP-1 staining patterns among NOM, OED, and OSCC samples
3. To calculate and compare the cIAP-1 labeling indices (LIs) in NOM, OED and OSCC samples
4. To assess the correlation between the cIAP-1 LI in OSCCs and the clinicopathological parameters or survival of OSCC patients.

# Literature Review

## Part 1: Introduction of oral squamous cell carcinoma

### Epidemiology in the world

Oral cancer is the fifth most common cancer in the world (Lingen et al. 2001) and accounts for approximately 4% of all cancers (Boyle et al. 1990). Among all oral malignancies, the oral squamous cell carcinoma (OSCC) was the most common type. In the United States, the annual number of new oral carcinoma cases is about 29,370 (Jemal et al. 2005). In Southeast Asian countries and India where the habit of areca quid (AQ) chewing is very popular, oral cancers are the most common forms of cancer and constitute 30-40% of all cancers (WHO meeting reporting, 1984). In Europe, USA, Australia and Japan, tobacco and alcohol are the two main carcinogens for oral precancerous lesions and oral cancers. In Taiwan, India, Sri Lanka, Papua New Guinea and Southeast Asia, the major etiologic agents associated with the development of either oral precancerous lesions or OSCCs are AQ, tobacco and alcohol.

### Epidemiology in Taiwan

In Taiwan, oral cancers ranks as the six most prevalent cancer in both sexes and account for the fourth most common cancer in males (Cancer registry annual report, ROC, 2007). There are two million people who habitually chew AQs in Taiwan. (Ko et al, 1995); approximately 80% of all oral cancer deaths are associated with this habit (Kwan 1976). Ko et al. (1995) found that the incidence of oral cancer in Taiwan is 123-fold higher in patients who smoke, drink alcohol, and chew AQs than in abstainers. The odds ratios of patients who indulge in at least two of the three habits are significantly elevated as compared with the odds ratios of patients with a single habit.

Furthermore, a statistically significant association between oral cancer and AQ chewing alone is also found (Ko et al. 1995).

## Etiology

The causes of OSCC are multifactorial and the oral carcinogenesis is regarded as a multistep process including initiation, promotion, and progression. The multistep carcinogenesis is defined by the development of oral carcinoma as a result of several separate events during the initiation or promotion of malignant transformation (Schwartz 2000). Oral carcinogenesis involves multiple genetic events that alter the normal functions of oncogenes and tumor suppressor genes (Williams 2000), leading to cell dysregulation with disruption in cell signaling, DNA-repair, and cell cycle (Bettendorf et al. 2004). Several studies have identified specific genetic alterations, such as some cytogenetic changes, oncogenes, tumor suppressor genes, and cell cycle regulators in oral carcinomas and in precancerous lesions of the oral cavity.

The cause of OSCC in Taiwan is closely related to AQ chewing. It had been estimated 200 to 400 million people chewed AQ worldwide (Zain et al. 1997) and only three drugs, i.e., nicotine, ethanol, and caffeine, are consumed more widely than AQ (Norton 1998). There are two million people who habitually chew AQ in Taiwan (Ko et al. 1992); approximately 80% of all oral cancer deaths are associated with this habit (Kwan 1976). The preparation of AQ varies from different geographic areas in the world, but it generally consists of betel nut (BN, *Areca catechu*), *Piper betle* leaf, and slaked lime with or without tobacco (Jeng et al. 1994). In Taiwan, AQ is chewed in two main ways. For aborigines, they use fresh areca nut wrapped simply in betel leaf (or betel vine by Yami Tribe, Orchid island) that is often (90%) lined with lime paste.

Another way is by the Chinese people that usually (97%) chew the areca nut ripe but not cured. That is, a lengthwise piece of unripe fruit from the *Piper betle*, lime paste, and some Chinese herbs or a secret formula are sandwiched between two halves of an areca nut (Ko et al. 1992). No tobacco is added in the AQ in Taiwan, which differs from the rest of Southeast Asia where the AQ chewing habit is also very popular.

The BN extract has been shown to be cytotoxic and genotoxic to cultured human buccal epithelial cells (Sundqvist et al. 1989). In the study of AQ marketed in Taiwan, Jeng et al. (1994) demonstrated several AQ constituents [arecoline, (+)-catechin, and extracts of inflorescence of *Piper betle* (IPB) and BN] can decrease cell survival and proliferation in a dose-dependent manner in human buccal mucosal fibroblasts. Furthermore, extracts of BN and IPB can also induce DNA strand break formation in a dose-dependent manner. In addition, arecoline is more cytotoxic than (+)-catechin and extracts of IPB and BN. They concluded that AQ contains not only genotoxic and cytotoxic agents, but also compounds which stimulate cell proliferation (Jeng et al. 1994).

There is a significant correlation of AQ chewing with the development of OSCC, leukoplakia, and oral submucous fibrosis (OSF) (Thomas and Kearsley 1993, Zain et al. 1997). Warnakulasuriya et al. (2002) found that AQ chewers (without tobacco) have an odds ratio of 5 for the development leukoplakia as opposed to an odds ratio of 1 in nonchewers (Warnakulasuriya et al. 2002). Ko et al. (1995) demonstrated that AQ chewing alone (without cigarette smoking and alcohol drinking) have a 28-fold higher potential to develop oral cancer. It has been shown that AQ chewing together with tobacco chewing or smoking is associated with an increased risk of oral cancer (Sanghvi 1981, IARC 1985). Of the Chinese AQ chewers in Taiwan, 86% are also a smoker and 75% are also a drinker (Ko et al. 1992). Ko et al. (1995) found that the

incidence of oral cancer in Taiwan is 123-fold higher in patients who smoke, drink alcohol, and chew BQ than in abstainers. The odds ratios of patients who indulge in at least two of the three habits are significantly elevated as compared with the odds ratios of patients with a single habit. In the study of 703 primary OSCC patients in southern Taiwan, Chen et al. (1999) demonstrated that those who have the habits of alcohol drinking, AQ chewing, and cigarette smoking have a 5.32-fold increased likelihood of death than nonusers.

## Clinical presentation

Squamous cell carcinoma (SCC) is the most common type of oral carcinoma. It accounts for about nine of every ten oral malignancies, and is a major cause of cancer morbidity and mortality. OSCC has a varied clinical presentation, including exophytic and endophytic masses. A majority of oral cancers are found to develop from oral premalignant lesions such as leukoplakia, erythroplakia, erythroleukoplakia, dysplasia, and carcinoma *in situ*. The malignant transformation rates of oral premalignant lesions are reported to be 1-7% for homogenous, thick leukoplakia, 4-15% for granular or verruciform leukoplakia, 18-47% for erythroleukoplakia, 4-11% for moderate dysplasia, and 20-35% for severe dysplasia (Neville et al. 1995). As OSCCs infiltrate and invade the underlying submucosal tissues, the mucosa becomes indurated or firm due to the mass effect of the tumorous epithelium and the reactive desmoplasia. Ulceration commonly occurs as invasive neoplasia progresses and invades into the underlying tissues. Induration and long-term unhealed ulceration invariably lead to a high degree of suspicion for an invasive carcinoma.

OSCC has historically been a male affliction with a marked association of these tumors with prolonged tobacco and alcohol use. In America, excluding the lip and tongue, the most common site for OSCC is the floor of the mouth (Krolls et al. 1976, Smith et al. 1979). However, the most frequent site for OSCC in Taiwan is the buccal mucosa. A retrospective study analyzing 703 cases of OSCC in southern Taiwan found an overwhelming male predominance (male: female =15:1) (Chen et al. 1999). The mean age of the oral cancer patients is 52 years. The peak age of OSCC patients declines from 50 to 59 years in the first six years (1985–1990) to 40 to 49 years in the next six years (1991–1996). OSCCs occur most commonly in the buccal mucosa with 37.4% of the total OSCCs being in this location. Most patients (346/703, 49.2%) have stage III cancer. The tongue is the most common site for patients without any oral habit (OH) (18/48, 37.5%). Furthermore, the age of the cancer patients without any OH was on average 6-12 years younger than patients who chewed AQs. The cancer stage significantly influences mortality: the 5-year survival rate in patients treated from 1985 to 1991 is 72% in those with stage I, 38.9% in those with stage II, 26.7% in those with stage III, and 11.8% in those with stage IV cancer (Chen et al. 1999).

## Histologic features

OSCC is characterized histopathologically by invasive islands, cords, strands or nests of malignant squamous epithelial cells. The invasion is represented by irregular extension of the lesional epithelium through the basement membrane into the underlying connective tissue, forming individual islands or nests independent from the surface epithelium. The invasive tumor cells may extend deeply into the adipose tissue, muscle, or bone in progressive lesions. The tumor cells usually show increased nuclear-cytoplasmic (N/C) ratio, hyperchromatism, cellular or nuclear pleomorphism,

mitotic figures, and variable amount of keratin formation. Desmoplastic reaction around the invasive tumor nests, lymphocytic or vascular permeation, or perineural invasion by the tumor cells can sometimes be present.

## Grading of squamous cell carcinoma

According to the level of squamous differentiation, OSCC is graded into well-, moderately-, and poorly-differentiated types. Well-differentiated OSCC is typically composed of large, closely apposed cell nests with prominent keratinization, often keratin pearl formation. Moderately-differentiated OSCC consists of smaller nests of more pleomorphic cells with increased mitotic activity and clear-cut but often less prominent squamous differentiation. Poorly-differentiated OSCC is composed of highly mitotically active cells growing singly or in small nests showing ragged or diffuse rather than "pushing" infiltration. Squamous differentiation is focal and often poorly developed.

## Our previous studies on oral cancers

The development of oral cancer is a multiple-step process that consists of a sequential accumulation of multiple genetic alterations. Tumorigenesis in the head and neck region seems to involve at least 6 to 10 independent genetic alterations (Renan 1993). Mutations and altered expression of oncogenes or tumor suppressor genes probably constitute the most frequent genetic changes associated with the process of carcinogenesis. Our previous studies have shown K-ras codon 12 mutations in 6 (18%) of 33 cases of OSCC (Kuo et al. 1994). By immunohistochemistry, we further found overexpression of ras p21 in 47 (92.2%) of 51 cases of OSCC (Kuo et al. 1995) and positive cyclin D1 imunostaining in 73 (83%) of 88 cases of OSCC (Kuo et al. 1999).

The patients with OSCCs containing more than 10% cyclin D1 positive cells have lower survival rate than those with OSCCs containing less than 10% cyclin D1 positive cells (Kuo et al. 1999). In addition, overexpression of p53 protein is found in 47 (58%) of 81 cases of OSCC, 30 (60%) of 50 cases of oral submucous fibrosis, 4 (40%) of 10 cases of oral leukoplakia, 7 (70%) of 10 cases of OED (Chiang et al. 1999, 2000). The patients with p53-positive OSCCs have lower survival rate than those with p53-negative OSCCs (Chiang et al. 1999). However, p53 mutations are only discovered in 2 (5.4%) of 37 OSCC cases (Kuo et al. 1999). Recently, we found that 31 of the 43 oral cancer patients (72%) have tumors with positive $p21^{WAF1}$ nuclear staining and 27 of the 43 patients (63%) have tumors with p53 nuclear staining (Kuo et al. 2002). The Kaplan-Meier analysis shows a significant correlation between $p21^{WAF1}$ protein overexpression and poor patient overall survival ($P = 0.049$). When p53 and $p21^{WAF1}$ are evaluated together, the 5-year overall survival was lowest in p53(+)-$p21^{WAF1}$(+) patients and highest in p53(-)-$p21^{WAF1}$(-) patients ($P = 0.057$). Therefore, combined evaluation of $p21^{WAF1}$ and p53 expressions may be useful in estimating the prognosis of patients with OSCC in Taiwan. Furthermore, we also found that the mean cyclin A labeling indices (LIs) in NOM, OED and OSCC samples are 7.0±3.1%, 12.1±3.9% and 21.3±12.3%, respectively. The cyclin A LI for OSCCs is significantly higher than that for NOM ($P = 0.002$) or OED ($P < 0.001$). In addition, a high cyclin A LI is found to correlate with advanced stage ($P = 0.0048$), larger tumor size ($P = 0.0017$), lymph node involvement ($P = 0.0006$), and cancer recurrence ($P < 0.0001$). The Kaplan-Meier analysis shows patients with tumors containing more than 15% cyclin A-positive cells have significantly shorter overall survival than those with tumors containing less than 15% cyclin A-positive cells ($P < 0.00001$). These results indicate that overexpression of cyclin A protein is associated with aggressive OSCC progression and poor prognosis for OSCC patients (Chen et al. 2003).

## Part 2: IAP family proteins

Inhibitor of apoptosis (IAP) family of proteins were first identified by Crook et al. in 1993 from baculoviruses. IAPs were shown to be involved in suppressing the host cell death response to viral infection. Ectopic expression of some baculoviral IAPs blocks apoptosis in mammalian cells, suggesting conservation of the cell death program among diverse species and commonalities in the mechanism used by the IAPs to inhibit apoptosis. Although the mechanism used by the IAPs to suppress cell death remains debated, several studies have provided insights into the biochemical functions of these intriguing proteins. Moreover, a variety of reports have suggested an important role for the IAPs in some human diseases. To date, eight human IAPs have been identified: cIAP-1, cIAP-2, NAIP, survivin, XIAP, Bruce, ILP-2, and livin.

## Structure

IAP family proteins are characterized by a novel domain of 疊70 amino acids termed the baculoviral IAP repeat (BIR), the name of which derives from the original discovery of these apoptosis suppressors in the genomes of baculoviruses by Lois Miller and her colleagues (Crook et al. 1993; Birnbaum et al. 1994). Up to three tandem copies of the BIR domain can occur within the known IAP family proteins of viruses and animal species. The BIR (baculoviral inhibitor of apoptosis repeat) domain is necessary to suppress apoptosis and is involved in the binding of IAPs to second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low pH (DIABLO) and caspases (Du et al. 2000).

Several of the mammalian, fly, and viral IAPs have a RING domain located near their carboxyl termini. With the exception of NIAP and survivin, human IAPs also contain a conserved sequence termed RING Zn finger at the carboxy-terminus. The presence of the RING Zn finger appears to be critical to the baculoviral IAP

anti-apoptotic function while it is dispensable for some (but not all) cellular IAP apoptotic inhibition. The human IAP family proteins cIAP-1, cIAP-2, and XIAP have been reported to retain anti-apoptotic function in the absence of their carboxy-terminal RING domains (Deveraux et al. 1997; Roy et al. 1997; Takahashi et al. 1998).

The human cIAP-1 and cIAP-2 proteins contain a caspase recruitment domain (CARD) located between the BIR and RING domains. The functional significance of this domain for the anti-apoptotic function of IAPs is largely untested, but amino-terminal fragments of human cIAP-1 and cIAP-2 that retain only the BIR domains are sufficient to block apoptosis, implying that the CARD domain is not absolutely required (Roy et al. 1997). cIAP-1 (also known as MIHB, hiap1, and BIRC2) and cIAP-2 (also known as MIHC, hiap2, and BIRC3) are structurally related to XIAP with three BIR domains and a RING finger. These IAPs were identified through the biochemical purification of proteins associated with the death receptor TNFR2.

Other domains of potential interest in IAP family proteins include a functionally intact ubiquitin-conjugating (UBC) domain in BRUCE, a large 528-kD BIR-containing protein (Hauser et al. 1998). Although it is not yet known whether the BRUCE protein suppresses apoptosis, BRUCE conceivably can provide a functional connection between apoptosis proteins and the ubiquitin proteasome pathway for protein degradation. The NAIP protein contains a P-loop consensus sequence similar to some ATP/GTP-binding proteins, but whether this IAP member binds purine nucleotides or requires this domain for apoptosis suppression remains undetermined (Roy et al. 1995). Taken together, the domain structure of IAPs suggests that the common unit, the BIR domain, can be linked with a variety of other motifs. These non-BIR motifs presumably either diversify the functions of IAPs or provide ways of regulating individual members or subgroups of the family of IAP proteins. So, c-IAP1 is a protein which has three BIR

domains, CARD domain, and carboxy-terminal RING domains.

## Gene location

The human *XIAP*, *c-IAP1*, *c-IAP2*, *NAIP*, and s*urvivin* genes have been assigned to chromosomal locations Xq25, 11q22-q23, 11q22-23, 5q13.1, and 17q25, respectively (Roy et al. 1995; Liston et al. 1996; Rajcan-Separovic et al. 1996; Ambrosini et al. 1998). Interestingly, the human *c-IAP1* and *c-IAP2* genes are located within 疊7 kbp of each other on 11q22-23 (Young et al. 1999).

## Part 3: Associated proteins--Caspase structure

Genetic analyses in the nematode *Caenorhabditis elegans* have identified two genes, ced-3 and ced-4, that are required for the execution of the cell death process (Yuan and Horvitz 1992; Yuan et al. 1993). The cloning of ced-3, a gene that encodes a protease with homology to mammalian interleukin-1b-converting enzyme, provided the first indication that cysteine proteases are critical components of the cell death machinery (Yuan et al. 1993). This observation leads to the identification of a growing family of cysteine proteases with homology to CED-3 that has been designated caspases (Alnemri et al. 1996). These cysteine proteases are synthesized in the cell as inactive precursors composed of four distinct domains: an amino-terminal domain of variable size (termed N-terminal polypeptide or prodomain), a large subunit, a small subunit, and a linker region between the large and small domains flanked by Asp residues (reviewed in Nicholson and Thornberry 1997). Activation of each caspase is induced by proteolytic cleavage between domains, resulting in the removal of the prodomain and linker regions, and assembly of the large and small subunits into an active enzyme complex. The mammalian cell death proteases have been divided into upstream

(initiator) and downstream (effector) caspases based on their sites of action in the proteolytic caspase cascade. In addition, caspases have different prodomains. Initiator caspases, but not effector caspases, have long prodomains containing structurally related protein modules that physically link these proteases to their specific activators. Two types of interaction modules have been detected in the prodomains of initiator caspases: death effector domain (DED) or caspase recruitment domain (CARD) (Boldin et al. 1996; Muzio et al. 1996; Hoffman et al. 1997). Nematode CED-3 and mammalian caspase-1, -2, -4, -5, -8, -9, -10, -11, -12 and -13 have prodomains with DEDs or CARDs. In contrast, caspase-3, -6, -7 and -14 have short prodomains. DEDs and CARDs in certain procaspases physically connect the initiator caspases with critical regulatory molecules via homophilic interactions. The role of initiator and effector caspases in the proteolytic cascade is consistent with the optimal recognition motifs of these caspases. Initiator caspases have substrate specificities that are similar to caspase recognition sites present in their own sequence (Thornberry et al. 1997), implying that these caspases can utilize autocatalysis for activation. Moreover, optimal caspase recognition sites for initiator caspases are present in the sequence of several effector proenzymes including procaspase-3 and procaspase-7, suggesting that these enzymes act downstream of initiator caspases in the proteolytic cascade (Thornberry et al. 1997).

## Caspase activation pathway

Caspases lie in a latent (zymogen) state in cells but become activated in response to a wide variety of cell death stimuli. Through a proteolytic cascade, caspases are functionally connected to each other, with upstream (initiator) caspases cleaving and activating downstream (effector) caspases (Salvesrm et al. 1997). At present, IAPs inhibit at least two of the major pathways for initiation of caspase activation: (*a*) the mitochondrial pathway with cytochrome *c*, and (*b*) the death receptor pathway with the

tumor necrosis factor (TNF) family of death receptors.

### *i. The Mitochondrial Pathway of Caspase Activation*

The intrinsic pathway for caspase activation is initiated by the release of cytochrome *c* from the mitochondria. Cytochrome *c* is normally sequestered between the inner and outer membranes of the mitochondria. In response to a variety of proapoptotic stimuli, cytochrome *c* is released into the cytosol (Kluck et al. 1997). Cytochrome *c* then binds and activates Apaf-1. Apaf-1 activates procaspase 9, which in turn cleaves procaspase 3 (Saleh et al. 1999 and Zou et al. 1997).

### *ii. The Death Receptor Pathway of Caspase Activation*

The death receptor pathway for caspase activation begins with the TNF family of cytokine receptors, which includes Fas (CD95), DR4 (Trail-R1), and TNF-R1 (CD120a). Death receptors are activated by ligand binding to the extracellular domain of the receptor. Once activated, death receptors recruit the death domain protein Fadd/Mort-1. When bound to the death receptor, Fas-associated death domain in turn binds caspase 8 (Chang et al. 1999), thereby forming the death-inducing signaling complex (DISC). As caspase 8 concentrates at the DISC, it dimerizes and thereby becomes active. Cleavage of caspase 8 enhances its stability in the dimerized form (Boatright et al. 2003). Active caspase 8 is released from the DISC into the cytosol, where it cleaves and activates the downstream effector caspases (Kang et al. 1999).

### *iii. Convergence Point and Cross-Talk*

The intrinsic and extrinsic pathways converge at the activation of downstream effector caspases such as caspase 3 and 7. Active caspase 3 then cleaves critical intracellular proteins to induce the final stages of cell death. Although the two caspase

pathways are presented as separate entities, cross-talk exists between them. For example, the two pathways cooperate to enhance apoptosis through Bid. Bid, a member of the BH3-only family of proapoptotic proteins, is cleaved and activated by caspase 8. When cleaved, Bid migrates to the mitochondria, where it promotes permeabilization of the mitochondrial membrane, cytochrome *c* release, and initiation of the intrinsic pathway of caspase activation (Tang et al. 2000, Kulik et al. 2001, and (Gross et al. 1999).

## Part 4: Associated proteins-Smac/DIABLO

Smac/DIABLO, which was first identified in 2000 by Du et al., is normally a mitochondrial protein but is released into the cytoplasm when cells undergo apoptosis. The IAP-inhibitory functions of the SMAC family of proteins are encoded in their NH2 terminus. Peptides corresponding to the seven NH2-terminal amino acids are capable of binding XIAP (Arnt et al. 2002). Mutation of the NH2-terminal alanine to glycine abolishes the ability of the SMAC peptide to bind IAPs and exerts its proapoptotic function (Liu et al. 2000). Smac/DIABLO has been demonstrated to promote activation of caspase-3, caspase-7 and caspase-9 by binding IAPs (e.g. XIAP, cIAP-1, cIAP-2, and survivin) and suppressing their inhibitory activity (Du et al. 2000).

## Part 5: Associated proteins-HraA2

HtrA2 belongs to the HtrA family of serine proteases and is well conserved from bacteria to humans (Faccio et al., 2000; Gray et al., 2000; Hu et al., 1998; Savopoulos et al., 2000). Human HtrA2 serine protease has extensive homology with bacterial HtrA and L56, another human HtrA, at the C terminus of its polypeptide (Hu et al., 1998). Because mature HtrA2 protein is released from the mitochondria in response to apoptotic stimuli, under normal conditions mature HtrA2 protein is found within the

intermembrane space, as are cytochrome c and Smac. The fact that the first four N-terminal amino acids of mature HtrA2 protein, AVPS, are very similar to those of the mature Smac protein, AVPI, provides evidence that the two proteins function in a similar way to inhibit IAPs (Chai et al. 2000).

## Part 6: Mechanisms of IAPs-mediated inhibition of apoptosis

The mechanism of apoptosis inhibition remains unclear, as IAPs appear to act through several mechanisms to repress apoptosis (Deveraux et al. 1998; Duckett et al. 1998).

### Caspase inhibition

IAPs can block apoptosis through their ability to inhibit specific caspases. XIAP, c-IAP-1, c-IAP-2 and survivin directly bind and inhibit caspases 3, 7 and 9. cIAP-1 was first found in 1995 by Rothe et al. as a factor that binds to TNF receptor associated factor-1 (TRAF-1) and TNF receptor associated factor-2 (TRAF-2) in the signaling pathway mediated by TNF receptor 2 (TNFR2) (Rothe et al. 1995). In this pathway, cIAP-1 is known to inhibit directly the activity of caspase-3, caspase-7 and procaspase-9 (Roy et al. 1997 and Deveraux et al. 1998).

### Signal transduction pathways

Recently, several IAP family members have been shown to regulate apoptosis in a caspase-independent manner through the mitogen-activated protein (MAP) Jun kinase 1 (JNK1) signal transduction pathway (Sanna et al. 2002). NAIP, as well as XIAP and livin, are able to activate JNK1, while c-IAP1, c-IAP2 and survivin are unable to do so. It becomes apparent that IAPs are involved in the signal transduction of JNK signaling pathways, especially in the context of inflammatory stimulation, and that their effect is

pro-survival. Yet, conflicting results raise doubt whether IAPs activate or inhibit JNK activation. Further research on the interaction of IAPs with proteins in JNK signaling pathways is needed to clarify some of these controversies.

## Ubiquitylation

Recently, growing evidence has proven the significance of the proteasome in apoptosis as well (Jesenberger et al. 2002). The targeted protein is labeled with covalent modification of 8 kDa ubiquitin molecules. The process is initiated by ubiquitin-activating enzyme (E1), while ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) actually attach the ubquitin. Labeled proteins are recognized by the proteasome and are degraded. Interestingly, RING finger proteins might function as an E3 ubiquitin ligase. XIAP and c-IAP1 undergo RING-domain-dependent autoubiquitylation, which in turn labels them for proteasomal degradation (Yang et al. 2000).

By doing so, they act to lower the apoptotic barrier, thus allowing the cell to undergo apoptosis. XIAP can target active caspase 3 to proteasomal degradation (Suzuki et al. 2001). In contrast to autoubiquitylation, the ubiquitinylation of caspase 3 can be considered as a mechanism to protect the cell from apoptosis by lowering the active caspases' effect.

## Part 7: Regulation of IAPs

Cellular levels and activity of IAPs are controlled by several mechanisms, including transcriptional regulation, via nuclear factor-κB (NFκB) (Wang et al. 1998 and Stehlik et al. 1998), and posttranslational modification, via proteasome degradation (Yang et al. 2000). NFκB was shown to control the transcription of several apoptosis regulating genes, including some IAPs such as XIAP, c-IAP-1, and c-IAP-2. The

activity of IAPs can be further modulated by 2 IAP-binding proteins, Smac/DIABLO (Du et al. 2000) and Omi/Htra2 (Suzuki et al. 2001), which prevent their association with caspases.

The BIR domain, in addition to its functional role, has a regulatory role as the binding domain of IAPs inhibitory proteins, such as Smac/DIABLO, Omi/Htra2, and XIAP associated factor 1 (XAF-1). Smac/DIABLO, which was first identified in 2000 by Du et al., is normally a mitochondrial protein but is released into the cytoplasm when cells undergo apoptosis. Smac/DIABLO is released from the mitochondria along with cytochrome c during apoptosis, and this protein functions to promote caspase activation by associating with the Apaf-1 apoptosome and inhibiting XIAP, cIAP-1, and cIAP-2. Smac/DIABLO has been demonstrated to promote activation of caspase-3, caspase-7 and caspase-9 by binding IAPs (e.g. XIAP, cIAP-1, cIAP-2, and survivin) and suppressing their inhibitory activity (Du et al. 2000 and Verhagen et al. 2000).

A serine protease called Omi/Htra2 is released from mitochondria and inhibits the function of XIAP by direct binding in a similar way to Smac. Moreover, when overexpressed extramitochondrially, HtrA2 induces atypical cell death, which is neither accompanied by a significant increase in caspase activity nor inhibited by caspase inhibitors, including XIAP. HtrA2 is a Smac-like inhibitor of IAP with a serine protease activity-dependent cell death-inducing effect.

Given the potential clinical utility of Smac-like molecules, efforts have been made to understand the physical interactions between Smac and IAPs. Structural studies have demonstrated that Smac binds XIAP at two distinct sites. The NH2 terminus of active Smac (residues 56–59) binds the BIR3 pocket of XIAP and competitively inhibits the BIR3 domain from binding caspase 9. Mutations in the BIR3 domain that prevent the binding to caspase 9 (*e.g.,* W310) also prevent the BIR3 domain from binding Smac, suggesting that the binding sites of Smac and caspase 9 overlap. However, the binding

sites are not identical because some mutations in BIR3 (*e.g.,* H343A) abolish the binding of BIR3 to caspase 9 but not to Smac (Liu et al.2000).

Smac full-length protein and NH2-terminal peptides also bind the BIR2 domain of XIAP, but with an affinity ~5- to 10-fold lower than that for BIR3. The mechanism by which Smac disrupts the association of BIR2 from caspase 3 is unclear, but it may be related more to steric hindrance than competitive binding (Liu et al.2000). Htra2 binds to the BIR3 domain of XIAP, but with weaker affinity than Smac (Li et al. 2002). In its active state, Htra2 exists as a trimer, and mutations that prevent trimer formation render HtrA2 inactive. In addition to inhibiting IAPs through binding the BIR3 pocket, Htra2 can also cleave and inactivate multiple IAPs including XIAP, cIAP-1, and cIAP-2, but not survivin (Yang et al. 2003). So, Smac/DIABLO and Omi/HtrA2 are inhibitor of cIAP-1.

Another fascinating example of a bi-directional effect is found in the intimate association of IAPs with caspases. IAPs inhibit caspases, yet this interaction comprises an intrinsic regulatory mechanism, as the caspases can cleave the IAPs. So far XIAP, cIAP-1, and most recently livin have been shown to undergo specific and functional cleavage by caspases. The cleavage of cIAP-1 occurs immediately after the BIR3 domain and produces a pro-apoptotic C-terminal fragment, which the RING domain is preceded by a spacer sequence of amino acids. The pro-apoptotic activity of cIAP-1 fragment, which does not contain BIR, is not surprising since RING domains of other baculoviral and mammalian IAPs are able to induce apoptosis when expressed without their BIR domains.

Interestingly, effector caspases 3, 6 and 7 and not upstream initiator caspases 8 and 9 are responsible for the specific proteolytic cleavage of the different IAP proteins. This is despite the fact that these anti-apoptotic factors are able to interact with both types of caspases. This might enable the cell to form a gradient of inhibition along the apoptotic

cascade. At the upstream level, IAPs inhibit caspase 9, which cannot cleave any IAP. Yet, once the cells are committed to apoptosis and downstream caspases are active, they can overcome IAP inhibition by a specific cleavage.

## Part 8: The emerging role of IAPs in cancers

Several studies demonstrated high levels of IAPs in early stages and even pre-malignant lesions, indicating an early role in these tumors. For example, XIAP, cIAP-1, cIAP-2 and survivin have been detected in prostatic intraepithelial neoplasia lesions (carcinoma *in situ*) (Krajewska et al. 2003). Survivin has been detected in pre-malignant lesions at similar levels observed in overt malignancy.

Intestinal epithelial cells undergo rapid proliferation at the base of the intestinal crypts, followed by differentiation, migration to the surface, and finally apoptosis. One of the earliest events in the development of colon cancer is upregulation of cyclooxygenase (COX)-2, which in turn increases cAMP production, which promotes growth and is anti-apoptotic. Remarkably, as cells migrate to the surface of the villi, a lowered level of cAMP is seen (Amelsberg et al. 1996) . Recently, c-IAP and livin expression are shown to be positively regulated by cAMP in colon epithelial cells (Nishihara et al. 2003).

One possible mechanism by which IAPs can promote tumorigenesis is by keeping mutated cells alive. As IAPs inhibit cell death, cells that suffered DNA breaks might be rescued, raising the possibility of malignant transformation (Herr et al. 2001). Several studies have demonstrated upregulation of certain IAPs in response to chemotherapy (Mansouri et al.2003) and radiation (Holcik et al. 2000). This upregulation have been shown to mediate cell resistance to apoptosis.

## Part 9: IAPs as therapeutic targets

Conventional cancer chemotherapies are cytotoxic chemicals that kill rapidly growing malignant cells. These drugs are indiscriminate toxins that lack cell selectivity. Therefore, they cause harmful effects to many types of healthy cells and consequently lead to potentially devastating and rarely even irreversible side effects to the treated patients. There has been an increasing interest in the development of anti-cancer targeted therapy based on rational drug design. The starting point for developing more selective and less harmful anti-cancer drugs is to single out targets associated with the signal transduction network within cells that are critical for proliferation, cell death, and angiogenesis. In this context, IAPs seem to fit ideally as a specific molecular target for cancer treatment. These cellular factors are differentially overexpressed in many types of malignant cells and not in their healthy counterparts.

### Antisense Oligonucleotides

One therapeutic strategy to inhibit IAPs uses antisense oligonucleotides to decrease the target IAP mRNA and subsequently decrease the protein. Antisense oligonucleotides inhibit IAPs by forming duplexes with intracellular native mRNA. The duplexes disrupt ribosome assembly and inhibit protein translation. More importantly, the mRNA-antisense oligonucleotides complex recruits RNase H enzymes that cleave the native mRNA strand while leaving the antisense oligonucleotide intact. The antisense oligonucleotide is then released back into the cytosol, where it is capable of inhibiting additional native mRNA (Crooke et al. 1998).

### Small molecule

Another strategy to block IAPs involves identifying small molecules that reverse IAP inhibition of caspases. A prototype for such a compound is the endogenous IAP

inhibitor Smac. Currently, most efforts are focusing on developing small molecules that inhibit XIAP because its crystal structure is known and the mechanism by which it inhibits caspases is well understood. Likewise, molecules that target IAPs such as XIAP or survivin will initially be used as single agents or in combination with low-dose chemotherapy in patients with relapsed or refractory disease. As more experience is gained, these targeted therapies will be used up-front in the treatment of *de novo* disease in combination with standard chemotherapy. In the future, when more small molecules that modulate the apoptosis cascade are developed, they will be used together to simultaneously target different molecular defects.

## Part 10: cIAP-1 expression in normal tissue

In an immunohistochemistry study, the epithelial cells, either from stratified or simple epithelia, showed positive staining for cIAP-1. Abundant cIAP-1 expression was found in the monostratified epithelial cells lining the crypts of Lieberkqhn of the large intestine, bronchi and bronchioli in the lung, the glands of the endometrium, the urothelium of the bladder, and the ducts of the salivary glands. Cells of endocrine origin, neurons, lymphocytes, germ cells, placental cells, plasma cells, and macrophages located in the interfollicular region of nodes and medullary cords of lymphoreticular organs are found to express cIAP-1. The vessels of the body are positive for cIAP-1, except in the colon, thymus, kidney, pituitary gland, and thyroid, where the smooth muscle layer is stained and the endothelium is negative or only weakly positive for cIAP-1 (Barbara et al. 2005). cIAP-1 is highly expressed in the kidney, small intestine, liver, and lung, but not in the central nervous system (Tanimoto et al. 2005).

## Part 11: cIAP-1 expression in cancers

Ferreira et al. (2001) assessed by immunohistochemistry the expression of the IAP

proteins cIAP-l, cIAP-2, and XIAP in tumors from 55 patients with advanced nonsmall-cell lung cancer (NSCLC) treated with chemotherapy, and correlated that with the observed response to chemotherapy, time to progression, and overall survival. The median expression of tumor cells for c-IAP-l, cIAP-2, and XIAP is 70%, 45%, and 25%, respectively. A correlation is observed between cIAP-l and cIAP-2 expression *(P* = 0.004), and between cIAP-l and XIAP expression *(P* = 0.013). However, no association was seen between the expression of these proteins and sex, age, tumor size, stage, histology, and grade of differentiation. Interestingly, expression of cIAP-l, cIAP-2, and XIAP does not predict response to chemotherapy. In addition, the expression of IAPs has no impact on the time to progression or overall survival of this group of patients, although the expression of cIAP-1 in nonsmall-cell lung cancer is common.

McEleny et al. (2002) assessed mRNA and protein expression of NAIP, cIAP-1, cIAP-2, XIAP, and survivin in the prostate cancer cell lines LNCaP, PC3, and DU145 by RNase protection assays, Western blotting, and immunohistochemistry. All prostate cell lines demonstrated expression of NAIP, cIAP-1, cIAP-2, XIAP, and survivin at the level of both mRNA and protein. NAIP mRNA is expressed only weakly, but expression is greatest in the DU145 and PC3 cells, and least in the LNCaP cells. This correlates well with the protein expression where PC3 and DU145 cells have the highest expression, with the lowest expression seen in the LNCaP cells. cIAP-2 mRNA and protein expression follows a similar pattern to NAIP. Survivin mRNA is expressed most strongly in the DU145 and PC3 cells, and most weakly in the LNCaP cells. Survivin protein expression is greatest in LNCaP cells, followed by PC3 cells and most weakly in the DU145 cells. There is no alteration in the expression of cIAP-1 and XIAP (both mRNA and protein) in the cell lines. Western blotting also confirms that androgen manipulation does not influence IAP expression. This study characterises the

expression of IAP in three of the most commonly used prostate cancer cells. IAP may make an important contribution to apoptotic resistance in patients with prostate cancer.

To identify the most likely target(s) for amplification at 11q21-q23, Imoto et al. (2001) determined the extent of the amplification by fluorescence *in situ* hybridization (FISH) and then analyzed 31 human ESCC (esophageal squamous cell carcinoma) cell lines for expression levels of 11 known genes and one uncharacterized transcript present within the 1.8-Mb commonly amplified region. Only cIAP-1, a member of the IAP (antiapoptotic) gene family, is consistently overexpressed in cell lines that show amplification. Additionally, the cIAP-1 protein is overexpressed in the primary tumors from which those cell lines have been established. The ESCC cell lines with cIAP-1 amplification are resistant to apoptosis induced by chemotherapeutic reagents. An increase in cIAP-1 copy number is also detected in 4 of 42 (9.5%) primary ESCC tumors that are not related to the cell lines examined. Because inhibition of apoptosis seems to be an important feature of carcinogenesis, cIAP-1 is likely to be a target for 11q21–23 amplification and may be involved in the progression of ESCC, as well as other malignancies. This study demonstrated that cIAP-1 is consistently overexpressed in cell lines that show amplification at 11q21-q23 region and the ESCC cell lines with cIAP-1 amplification are resistant to apoptosis induced by chemotherapeutic reagents.

Because amplification of 11q22 has been implicated in other malignancies also, including cervical squamous cell carcinomas (CSCCs), Imoto et al. (2002) attempted to correlate amplification and overexpression of cIAP-1 with radiation sensitivity in CSCC-derived cell lines and primary CSCC tumors. They found that 2 of 9 cell lines show amplification and consistent overexpression of cIAP-1, as well as significant resistance to radiation-induced cell death as compared with cell lines showing no cIAP-1 amplification. Immunohistochemical analysis of 70 primary CSCCs from patients treated only with radiotherapy demonstrated that both overall survival and local

recurrence-free survival are significantly poorer among patients with tumors showing high levels of nuclear cIAP-1 staining than among patients whose tumors revealed little or no nuclear cIAP-1. Multivariate analysis showed nuclear cIAP-1 staining to be an independent predictive factor for local recurrence-free survival after radiotherapy among patients with CSCC. These findings demonstrate that cIAP-1 may play an important role in the development/progression of this disease and that cIAP-1 can be a novel predictive marker for resistance to radiotherapy in individual CSCC patients.

Krajewska et al. (2003) examined the expression of four members of the IAP family (cellular inhibitor of apoptosis protein 1, cellular inhibitor of apoptosis protein 2, X chromosome-linked IAP, and survivin) by immunohistochemistry and immunoblotting in human prostate cancers and in prostate tissues from transgenic mice expressing SV40 large T antigen under control of a probasin promoter. Tumor-associated elevations in the levels of all four IAP family members are common in prostate cancers of both humans and mice, suggesting concomitant up-regulation of multiple IAP family proteins. Compared with normal prostatic epithelium, increased IAP expression is often evident even in prostatic intraepithelial neoplasia lesions (carcinoma *in situ*), suggesting that deregulation of IAP expression occurs early in the pathogenesis of prostate cancer. This study demonstrates that tumor-associated elevations in the expression of several IAP family proteins occur as a frequent and early event in the etiology of prostate cancer.

Kempkensteffen et al. (2006) examined the expression of cIAP-1 and cIAP-2 by real-time RT-PCR in renal cell carcinoma (RCC) and corresponding normal tissue samples obtained from a cohort of 127 RCC patients (median follow-up: 48 months) undergoing surgical treatment. Expression data are correlated to histopathological variables and outcome. Overexpression of cIAP-1 and cIAP-2 occurs in most RCC specimens ($p < 0.001$), but 20% of the patients have lower cIAP levels in malignant

than in normal tissues. The cIAP-1 expression correlates with the tumor stage, levels being higher in pT1 tumors than in advanced pathological stages ($p < 0.002$). Decreased cIAP-1 expression in RCC relative to paired normal samples predicts an abbreviated time to recurrence (hazard rate 2.96; 95% CI: 1.23–7.09) and tumor-specific survival (hazard rate 2.78; 95% CI: 1.22–6.38) irrespective of the tumor stage and grade. The prognostic effect of cIAP-1 is most pronounced in patients with pT3 disease (log rank test $p < 0.001$). The results of univariate and multivariate analysis suggest a prognostic value of cIAP-1 expression for RCC patients, downregulation indicating an aggressive, potentially lethal phenotype.

## Part 12: cIAP-1 expression in OSCC

In order to clarify the subcellular localization of cIAP-1 and to investigate its clinicopathological significance in head and neck SCCs (HNSCCs), Tanimoto et al. (2005) examined cIAP-1 expression in four oral SCC cell lines by immunocytochemistry and Western blot. Expressions of nuclear and cytoplasmic cIAP-1, caspase-3, and Smac/DIABLO are also examined immunohistochemically in 57 cases of the HNSCCs. cIAP-1 expression is detected in HSC-2, HSC-3, and HSC-4 cells by immunohistochemistry and Western blot. In HSC-2 and HSC-4 cells, cIAP-1 is detected in both the nuclear and cytoplasmic fractions. Nuclear cIAP-1 expression is positive in 17 (30%) of 57 HNSCCs, is correlated with lymph node metastasis ($P = 0.020$) and advanced disease stage ($P = 0.032$), and tends to be correlated with poor patient prognosis ($P = 0.059$). Cytoplasmic cIAP-1 expression shows similar but weaker clinicopathological correlations. Nuclear cIAP-1 expression is inversely correlated with caspase-3 expression, but is correlated with Smac/DIABLO expression. Nuclear cIAP-1 expression appears to be a useful marker for predicting poor patient prognosis in HNSCCs, and may play roles in HNSCCs through the signaling pathway

mediated by Smac/DIABLO and caspase-3.

# Materials and Methods

## Part 1: patients and specimens

After approval by the Hospital Review Board, we obtained formalin-fixed, paraffin-embedded tissue blocks from 73 patients (63 men and 10 women, mean age 53 years, range 31-76 years) with oral squamous cell carcinoma (OSCC), 19 patients (17 men and 2 women, mean age 53 years, range 33-70 years) with severe oral epithelial dysplasia (OED), 34 patients (28 men and 6 women, mean age 52 years, range 26-91 years) with moderate OED, 23 patients (21 men and 2 women, mean age 49 years, range 25-80 years) with mild OED, and 31 patients (22 men and 9 women, mean age 35 years, range 18-65 years) with normal oral mucosa (NOM). Diagnosis of OSCC and OED was based on histological examination of hematoxylin and eosin (H&E)-stained tissue sections. All patients received total surgical excision of their lesions of OSCC and OED at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, Taipei, Taiwan during the period from 1995 to 2005. Specimens were obtained from total surgical excision of the lesions. If lymph nodes were diagnosed as positive for OSCCs, neck dissection and post-operative radiation therapy were also included in the treatment protocol. Of the 73 cases of OSCC, 41 (56%) were buccal mucosa, 18 (25%) tongue, 8 (11%) gingiva, 4 (5%) palate, and 2 (3%) floor of the mouth cancers. The TNM status and clinical stages of OSCCs at initial presentation were determined according to the UICC convention (Sobin and Wittekind 1997). None of the patients had received any form of tumor-specific therapy before total surgical excision of the lesion.

Histological features of OSCC were further classified into three different types (well-, moderately-, and poorly-differentiated SCC). Of the 73 OSCC cases, there were 64 (88%) well- and 9 (12%) moderately-differentiated OSCCs.

The oral habit data were available for all 73 OSCC patients. Details of patients' oral habits (OHs), including daily/weekly consumption of areca quid (AQ), cigarette, and alcohol as well as the duration of these habits, were recorded. OSCC patients were defined as AQ chewers when they chew 2 or more AQs daily for at least one year, as cigarette smokers when they smoked every day for at least one year and consumed more than 50 packs of cigarettes per year, and as alcohol drinkers when they drank more than four days and consumed more than 20 g of pure alcohol per week for at least one year. According to these definitions, 57 (55 men and 2 women) were AQ chewers, 59 (56 men and 3 women) were smokers, and 50 (48 men and 2 women) were drinkers.

The biopsy specimens of NOM were obtained during extraction of impacted permanent lower third molars or during secondary stage of implantation surgery after obtaining informed consent. All of these subjects had none of the oral habits and oral mucosal diseases. The NOM sections showed normal stratified squamous epithelium with or without keratinization. Sections of cervical aquamous cell carcinoma were used as positive controls (Imoto et al. 2002) and Tris-buffered saline (TBS) instead of primary antibody was used for negative controls.

## Part 2: Immunohistological staining for cIAP-1

All the specimens for immunohistochemical (IHC) staining were fixed in 10% neutral formalin, embedded in paraffin, and cut in serial sections of 5 μm. IHC staining was performed using a peroxidase-labeled streptavidin-biotin technique. Briefly, tissue sections were deparaffinized, rehydrated, and then heated in a plastic slide holder (Dako, Copenhagen, Denmark) containing 0.01 M citrate buffer (pH = 6) in a microwave oven for 10 min to retrieve antigenicity. The endogenous peroxidase activity was blocked by immersing the sections in 3% $H_2O_2$ in methanol for 20 min. After washing in 10 mM TBS, pH 7.4, sections were incubated with 10% normal goat serum to block

non-specific binding. Sections were then incubated overnight at 4℃ with 1:100 dilution of anti-cIAP-1 polyclonal rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After washing in TBS, sections were treated with biotinylated goat anti-rabbit IgG, and subsequently with a streptavidin-peroxidase conjugate (Dako). The 0.02% diaminobenzidine hydrochloride (DAB, Dako) containing 0.03% $H_2O_2$ was used as chromogen to visualize the peroxidase activity. The preparations were lightly counterstained with hematoxylin, mounted with Permount, and examined by light microscopy.

To evaluate the IHC staining, the sections were initially scanned at low power, at least three high-power fields were then chosen randomly, and at least 1,000 benign, dysplastic or malignant epithelial cells were counted for each case. A brown cytoplasmic staining was considered positive for each cell. The cIAP-1 labeling indices (LIs) were counted as a ratio of immunostaining-positive cells to the total number of cells counted. The staining intensity (SI) was also recorded and graded as 0, negative; 1, weak; 2, moderate; and 3, strong. An eyepiece graticule was used to ensure that all cells were evaluated once only. The cIAP-1 LIs in OSCC samples were calculated to assess whether the cIAP-1 LI in OSCC samples was a biomarker for prediction of the progression of OSCC and of the patients' survival. Each of these assessments was carried out by two investigators independently. The sections with an interobserver variation of more than 10% were reassessed by using a double-headed light microscope to achieve consensus. In this study, the interobserver reproducibility was 92%. The slides with discrepant assessments were reevaluated, and a consensus was reached in all cases.

## Part 3: Statistical analysis

The mean cIAP-1 LIs for OSCC, OED (severe, moderate, mild), and NOM

samples were compared first among 3 groups by analysis of variance (ANOVA) and then between any 2 groups by Student's *t*-test. The correlation between cytoplasmic cIAP-1 LIs in OSCCs and clinicopathological parameters of OSCC patients was analyzed by Student's *t*-test or ANOVA, where appropriate. The expression of cIAP-1 in OSCCs was also correlated with the AQ chewing, cigarette smoking, and alcohol drinking oral habits. Cumulative survival was analyzed with the Kaplan-Meier product-limit method. The duration of survival was measured from the beginning of treatment to the time of death or the last follow-up. Comparison of cumulative survival between groups was performed using the log-rank test with the Statisca program (StatSoft Inc., Tulsa, OK, USA). Univariate and multivariate survival analyses were performed with Cox proportional hazard regression model to assess additional prognostic values of the different variables using SAS 9.1 (SAS Institute Inc., Morrisville, NC, USA). A *P*-value of less than 0.05 was considered statistically significant.

# Results

## Part 1: Expression of cIAP-1 and mean LIs for OSCC, OED, and NOM samples

In general, the cytoplasmic cIAP-1 staining could be observed in normal, dysplastic and malignant epithelial cells. However, OSCC samples had the stongest cytoplasmic cIAP-1 SI, followed by OED and NOM samples. Although the cytoplasmic cIAP-1 SI was weak in nearly all normal oral epithelial cells, the cells of the upper half layer of the epithelium had a slightly denser staining than the lower half layer of the epithelium. In OED and OSCC samples, a moderate to strong cytoplasmic cIAP-1 staining was found in partial to nearly all dysplastic and malignant epithelial cells (Figure 1 A to F).

The mean cytoplasmic cIAP-1 LIs increased significantly from NOM (23 ± 22%) through OED (50 ± 25%) to OSCC samples (73 ± 17%) ($P$=0.000, Table 1). The detailed statistical data for comparison between any two groups are shown in Table 1.

## Part 2: Correlation between the mean cIAP-1 LI in OSCCs and clinicopathological parameters of OSCC patients

Correlation between cytoplasmic cIAP-1 LI in OSCC samples and clinicopathological parameters of OSCC patients is shown in Table 2. A significant correlation was found between the higher mean cytoplasmic cIAP-1 LI and OSCCs with positive lymph node metastasis ($P$ = 0.000) or more advanced clinical stages ($P$ = 0.045) (Table 2). However, the cytoplasmic cIAP-1 LI in OSCC samples was not significantly associated with patients' age and gender, cancer location, T status, histological differentiation of OSCCs, and cancer recurrence (Table 2). Univariate analysis performed by Cox proportional hazard regression model identified positive

lymph node metastasis ($P$ = 0.0105) and advanced clinical stage ($P$ = 0.0523, marginal significance) as correlating with poor survival. However, none was identified as an independent unfavorable prognosis factor by multivariate analyses with Cox proportional hazard regression model (Table 4).

## Part 3: Correlation between the mean cytoplasmic cIAP-1 LI in OSCCs and oral habits of OSCC patients

The correlation between cytoplasmic cIAP-1 LI in OSCCs and oral habits of OSCC patients is shown in Tables 3. No significant association was found between cytoplasmic cIAP-1 LI in OSCCs and AQ chewing, cigarette smoking, or alcohol drinking habits of OSCC patients.

## Part 4: Correlation between cytoplasmic cIAP-1 LI in OSCCs and survival of OSCC patients

The correlation between cytoplasmic LI in OSCCs and survival of OSCC patients was analyzed with Kaplan-Meier product-limit method. No significant association was found between cytoplasmic cIAP-1 LI in OSCCs and survival of all 73 OSCC patients ($P$ = 0.63154) (Figure 2).

# Discussion

Suppression of apoptosis is believed to contribute to tumorigenesis by abnormally prolonging cellular life span, enhancing growth factor-dependent cell survival and resistance to immunobased cytotoxicity, and allowing cells to miss cell cycle checkpoints that would normally induce apoptosis. This study showed a significant increase in the mean cytoplasmic cIAP-1 LI from NOM through OED to OSCC samples. We found that although NOM samples expressed a relatively small amount of cIAP-1 protein, there was a small but significant elevation in the cytoplasmic cIAP-1 expression from NOM to mild and moderate OED. Furthermore, a big and significant jump in the cytoplasmic cIAP-1 expression was noted from moderate to severe OED. However, no significant difference in the mean cytoplasmic cIAP-1 LI was found between severe OED and OSCC samples. These findings suggest that accumulation of enough amount of cIAP-1 is necessary for the transformation of normal to moderately dysplastic epithelium and for the transformation of moderately to severely dysplastic epithelium. In addition, there may be no significant change in the cIAP-1 level during the transformation from the severely dysplastic oral lesion to an oral cancer.

This study showed a significant increase in the cIAP-1 protein expression in OSCC than in NOM samples. Previous studies also demonstrate a higher expression of the cIAP-1 protein in cancers than in corresponding normal epithelia in lung (Ferreira et al. 2001, Dai et al. 2003), prostate (Krajewska et al. 2003), and early stage colorectal carcinoms (Krajewska et al. 2005). The significant increase in the cIAP-1 protein in cancers than in corresponding normal epithelia indicates that cIAP-1 may be a good biomarker for certain types of human malignancies and may play an important role in human carcinogenesis. Actually, overexpression of the cIAP-1 protein has been shown

in a variety of human carcinomas, including ovarian (Li et al. 2001), lung (Ferreira et al. 2001, Dai et al. 2003), prostate (Krajewska et al. 2003), cervical (Imoto et al. 2002, Liu et al. 2001), head and neck (Tanimoto et al. 2005), esopgageal (Imoto et al. 2001), and early stage colorectal carcinoms (Krajewska et al. 2003).

This study demonstrated a slightly higher cIAP-1 expression in T3 and T4 than in T1 and T2 oral cancers, although the difference was not significant. The cIAP-1 protein can suppress apoptosis induced by a variety of triggers; this finally results in prolonging the life span of cancer cells and tumor growth. Therefore, it is not difficult to explain why there is a slightly higher expression of cIAP-1 in larger than in smaller oral cancers.

Previous studies demonstrated a significant association of higher cIAP-1 expression with positive lymph node metastasis in head and neck SCCs (Tanimoto et al. 2005). This study also showed a higher cytoplasmic cIAP-1 LI in OSCCs with nodal metastases than OSCCs without nodal metastases. c-IAP-1 can directly bind and inhibit caspases 3, 7 and 9 (Roy et al. 1997, Deveraux et al. 1998). Because inhibition of apoptosis can not only prolong the life span of cancer cells but also promote cancer cell metastasis, it is easy to understand why there is a higher rate of lymph node metastasis in human cancers with elevated expression of cIAP-1.

This study showed a positive association of cIAP-1 overexpression with higher T and N statuses in OSCCs. Because higher T and N statuses always result in a more advanced clinical stage of OSCC, it is not difficult to explain why OSCCs with the higher expression of cIAP-1 are prone to have the more advanced clinical stages. Indeed, high expression of cIAP-1 is significantly associated with an advanced clinical stage in head and neck SCCs (Tanimoto et al. 2005).

Tanimoto et al. (2005) used immunocytochemistry and Western blot analysis to study cIAP-1 expression in head and neck SCC cell lines and tissue samples. They

found both nuclear and cytoplasmic cIAP-1 expressions in head and neck SCC cell lines and tissue samples. In addition, nuclear cIAP-1 expression is a better biomarker than cytoplasmic cIAP-1 expression for prediction of lymph node metastasis and clinical stage of head and neck SCCs and patients' prognosis. In this study, however, only cytoplasmic cIAP-1 staining was found in our NOM, OED and OSCC specimens. We suggest that the difference in intracellular localization of the cIAP-1 protein in various human carcinomas is due to the use of different antibodies against different epitopes of the cIAP-1 protein.

Imoto et al. (2002) showed that both overall survival and local recurrence-free survival are significantly poorer among cervical SCC patients showing high levels of nuclear cIAP-1 staining than among those whose tumors reveal little or no nuclear cIAP-1. Multivariate analysis also showed nuclear cIAP-1 staining to be an independent predictive factor for local recurrence-free survival after radiotherapy among cervical SCC patients. Furthermore, a significantly higher cIAP-1 expression in head and neck SCCs is also related to a poorer patient prognosis (Tanimoto et al. 2005). However, this study did not reveal a significant correlation between the cytoplasmic cIAP-1 expression in OSCCs and patients' overall survival. In addition, Kempkensteffen et al. (2006) demonstrated that decreased cIAP-1 expression in renal cell carcinomas predicts an abbreviated time to recurrence and tumor-specific survival irrespective of the tumor stage and grade.

Previous study suggested that cIAP-1 and cIAP-2 are nuclear shuttling proteins. The interaction of cIAP-1 and cIAP-2 with TRAF-2 plays an important role in their movement into the nucleus (Vischioni et al. 2003). cIAP-1 and cIAP-2 can shuttle between nucleus and cytoplasm using a nuclear import pathway that remains to be identified and the CRM1-dependent nuclear export pathway. Binding to TRAF-2 leads to the retention of the IAPs in the cytoplasm by preventing their translocation into the

nucleus (Vischioni et al. 2003). The processing of procaspase-9 by caspase-3 has been suggested to occur not only in mitochondria but also in the nucleus (Ritter et al. 2000). Therefore, it might be possible that caspase-9 processing, which is necessary for the execution of apoptosis, is inhibited significantly by cIAP-1 in the nucleus. The nuclear localization of caspase-3 and procaspase-9, targets for the anti-apoptotic effect of cIAP-1, suggests that binding or interaction between cIAP-1 and caspases may occur in the nucleus (Ritter et al. 2000).

Our results showed that the cIAP-1 is universally expressed in the cytoplasm of normal, dysplastic and malignant epithelial cells. The cytoplasmic expression of cIAP-1 significantly increased from NOM through OED to OSCC samples, and is related to N status and clinical staging of OSCCs. The increased expression of cIAP-1 protein is an early event in oral carcinogenesis and cIAP-1 may be a biomarker for OSCCs. Measuring the amount of cytoplasmic expression of cIAP-1 in OSCC samples may predict the oral cancer progression in Taiwan.

.

# Conclusions

1. The increased expression of cIAP-1 is an early event in oral carcinogenesis in Taiwan.
2. cIAP-1 may be a biomarker for OSCCs.
3. There is a significant association of higher cytoplasmic cIAP-1 espression in OSCCs with lymph node metastasis and more advanced clinical stages.
4. Measuring the amount of cytoplasmic expression of cIAP-1 in OSCC samples may predict the oral cancer progression in Taiwan.

# References


Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. Human ICE/CED-3 Protease Nomenclature. *Cell* 1996;87: 171-172.

Ambrosini G, Adida C, Sirugo G, and Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J. Biol. Chem.* 1998; 273: 11177–11182.

Amelsberg M, Amelsberg A, Ainsworth MA, Hogan DL, Isenberg JI. Cyclic adenosine-3',5'-monophosphate production is greater in rabbit duodenal crypt than in villus cells. *Scand J Gastroenterol* 1996;31:233–9.

Arnt CR, Chiorean MV, Heldebrant MP, Gores GJ, Kaufmann SH. Synthetic Smac/ DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAPand cIAP1 in situ. *J Biol Chem* 2002;277:44236–43.

Berglund H, Olerenshaw D, Sankar A, Federwisch M, McDonald NQ, Driscoll PC. The three-dimensional solution structure and dynamic properties of the human FADD death domain. *J Mol Biol* 2000;302:171–88.

Birnbaum, MJ, Clem RJ, Miller LK. An apoptosisinhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J Virol* 1994;68**:** 2521–2528.

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS. A unified model for apical caspase activation. *Mol Cell* 2003;11:529–41.

Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a Novel MORT1/FADD-Interacting Protease, in Fas/APO-1- and TNF Receptor–Induced Cell Death. *Cell* 1996;85: 803-815.

Chai, J, Du, C, Wu, JW, Kyin, S, Wang, X, Shi, Y. Structural and biochemical basis of

apoptotic activation by Smac/ DIABLO. *Nature* 2000;*406*: 855–862.

Chang H, Yang X, Baltimore D. Dissecting Fas signaling with an altered-specificity death-domain mutant: requirement of FADD binding for apoptosis but not Jun N-terminal kinase activation. *Proc Natl Acad Sci USA* 1999;96:1252–6.

Chen HM. Yen-Ping Kuo M. Lin KH. Lin CY. Chiang CP. Expression of cyclin A is related to progression of oral squamous cell carcinoma in Taiwan. *Oral Oncology.* 2003 Jul;39(5):476-82.

Chen YK. Huang HC. Lin LM. Lin CC. Primary oral squamous cell carcinoma: an analysis of 703 cases in southern Taiwan. *Oral Oncology.* 1999 Mar;35(2):173-9,

Chiang CP. Huang JS. Wang JT. Liu BY. Kuo YS. Hahn LJ. Kuo MY. Expression of p53 protein correlates with decreased survival in patients with areca quid chewing and smoking-associated oral squamous cell carcinomas in Taiwan. *Journal of Oral Pathology & Medicine.* 1999 Feb;28(2):72-6.

Clem, RJ, Miller LK. Control of programmed cell death by the baculovirus genes p35 and IAP. *Mol. Cell. Biol.*1994;14: 5212–5222.

Crook NE, Clem RJ, Miller LK, An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol.* 1993;67:168–174.

Crooke ST. Molecular mechanisms of antisense drugs: RNase H. *Antisense Nucleic Acid Drug Dev* 1998;8:133–4.

Dai Z, Zhu WG, Morrison CD, Brena RM**,** Smiraglia DJ, Raval A, Wu YZ, Rush LJ, Ross P, Molina JR, Otterson GA, Plass C. A comprehensive search for DNA amplification in lung cancer identifies inhibitors of apoptosis cIAP1 and cIAP2 as candidate oncogenes. *Hum Mol Genet* 2003;12:791 - 801.

Deveraux QL, Reed JC. IAP family proteins—suppressors of apoptosis. *Genes Dev* 1999;13:239–52.

Deveraux, QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor

of cell death proteases. *Nature* 1997;388: 300–303.

Deveraux, QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula M, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J.*1998;17: 2215–2223.

Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition, *Cell* 2000;102:33–42.

Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, Shiels H, Hardwick JM, Thompson CB. *EMBO J*. 1996;15: 2685-2694.

Faccio L, Fusco C, Chen A, Martinotti S, Bonventre JV, Zervos AS. Characterization of a novel human serine prote-Herrase that has extensive homology to bacterial heat shock endoprotease HtrA and is regulated by kidney ischemia. *J. Biol. Chem*. 2000;*275*: 2581–2588.

Ferreira CG, van der Valk P, Span SW, Jonker JM, Postmus PE, Kruyt FA, et al. Assessment of IAP (inhibitor of apoptosis) proteins as predictors of response to chemotherapy in advanced non-small-cell lung cancer patients. *Ann Oncol* 2001;12:799–805.

Gray CW, Ward RV, Karran E, Turconi,S, Rowles A, Viglienghi D, Southan C, Barton A, Fantom KG, West A, Savopoulos J, Hassan NJ, Clinkenbeard H, Hanning C, Amegadzie B, Davis JB, Dingwall C, Livi GP, Creasy CL. Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. *Eur. J. Biochem*. 2000;*267*: 5699–5710.

Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not

TNF-R1/Fas death. *J Biol Chem* 1999;274:1156–63.

Hauser HP, Bardroff M, Pyrowolakis G, Jentsch S. A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J. Cell Biol.*1998;141: 1415–1422.

Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. *Blood* 2001;98:2603–14.

Holcik M, Yeh C, Korneluk RG, Chow T. Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. *Oncogene* 2000;19:4174–7.

Hu SI, Carozza M, Klein M, Nantermet P, Luk D, Crowl RM. Human HtrA, an evolutionarily conserved serine protease identified as a differentially expressed gene product in osteoarthritic cartilage. *J. Biol. Chem*. 1997;273: 34406–34412.

Imoto I, Tsuda H, Hirasawa A, Miura M, Sakamoto M, Hirohashi S, Inazawa J. Expression of cIAP1, a target for 11q22 amplification, correlates with resistance of cervical cancers to radiotherapy. *Cancer Res*.2002;62: 4860–4866.

Imoto I, Yang ZQ, Pimkhaokham A, Tsuda H, Shimada Y, Imamura M, Ohki M, Inazawa J. Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. *Cancer Res*.2001;61: 6629–6634.

Jeng JH. Kuo ML. Hahn LJ. Kuo MY. Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. *Journal of Dental Research.* 1994 May ;73(5):1043-9.

Jesenberger V, Jentsch S. Deadly encounter: ubiquitin meets apoptosis. *Nat Rev Mol Cell Biol* 2002;3:112–21.

Kang J, Schaber M, Srinivasula S, Srinivasula M, Alnemri ES, Litwack G, Hall DJ, Bjornsti MA. Cascades of mammalian caspase activation in the yeast Saccharomyces cerevisiae. *J Biol Chem* 1999;274:3189–98.

Kempkensteffen C, Hinz S, Christoph F, Kollermann J, Krause H, Schrader M,

Schostak M, Miller K, Weikert S. Expression parameters of the inhibitors of apoptosis cIAP1 and cIAP2 in renal cell carcinomas and their prognostic relevance. *Int. J. Cancer* 2006;120: 1081–1086

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* (WashDC) 1997;275:1132–6.

Krajewska M, Krajewski S, Banares S, Huang X, Turner B, Bubendorf L, Kallioniemi OP, Shabaik A, Vitiello A, Peehl D, Gao GJ, Reed JC. Elevated expression of inhibitor of apoptosis proteins in prostate cancer. *Clin Cancer Res* 2003;9:4914–25.

Krajewska M, Kim H, Kim C, Kang H, Welsh K, Matsuzawa SI, Tsukamoto M, Thomas RG, Assa-munt N, Zhe P, Suzuki K, Perucho M, Krajewski S, Reed JC. Analysis of apoptosis protein expression in early-stage colorectal cancer suggests opportunities for new prognostic biomarkers. *Clin Cancer Res* 2005;11:5451 - 61.

Kulik G, Carson JP, Vomastek T, Overman K, Gooch BD, Srinivasula S, Alnemri E, Nunez G, Weber MJ. Tumor necrosis factor alpha induces BID cleavage and bypasses antiapoptotic signals in prostate cancer LNCaP cells. *Cancer Res* 2001;61:2713–9.

Kuo MYP, Huang JS, Kok SH, Kuo YS, Chiang CP, Prognostic role of p21WAF1 expression in areca quid chewing and smoking-associated oral squamous cell carcinoma in Taiwan. *Journal of Oral Pathology & Medicine.* 2002 Jan :*31(1):16-22,*.

Kuo MYP. Lin CY. Hahn LJ. Cheng SJ. Chiang CP. Expression of cyclin D1 is correlated with poor prognosis in patients with areca quid chewing-related oral squamous cell carcinomas in Taiwan. *Journal of Oral Pathology & Medicine.* ;1999 Apr:*28(4):165-9,*

LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene* 1998;17:3247–59.

Li J, Feng Q, Kim JM, Schneiderman D, Liston P, Li M, Vanderhyden B, Faught W, Fung MFK, Senterman M, Korneluk RG, Tsang BK. Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins. *Endocrinology* 2001;142:370 - 80.

Li W, Srinivasula SM, Chai J, Li P, Wu JW, Zhang Z, Alnemri ES, Shi Y. Structural insights into the pro-apoptotic function of mitochondrial serine protease HtrA2/Omi. *Nat Struct Biol* 2002;9:436–41.

Lin CY, Hung HC, Chiang CP, Kuo MYP. Survivin expression predicts poorer prognosis in patients with areca-quid chewing-related oral squamous cell carcinoma in Taiwan. *Oral Oncol* 2005;41:645-54.

Lin PY, Yu CH, Wang JT, Chen HH, Cheng SJ, Kuo MYP, Chiang CP. Expression of hypoxia-inducible factor-1α is significantly associated with the progression and prognosis of oral squamous cell carcinomas in Taiwan. *J Oral Pathol Med* 2008;37:18-25.

Liu SS, Tsang BK, Cheung AN, Xue WC, Cheng DK, Ng TY, Wong LC, Ngan HY Anti-apoptotic proteins, apoptotic and proliferative parameters and their prognostic significance in cervical carcinoma. *Eur J Cancer* 2001;37:1104- 10.

Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: there is more to life than Bcl2. *Oncogene* 2003;22:8568–80.

Liston, P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, Farahani R, McLean M, Ikeda J, MacKenzie A, Korneluk RG. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 1996;379: 349–353.

Liu Z, Sun C, Olejniczak ET, Meadows RP, Betz SF, Oost T, Herrmann J, Wu JC, Fesik

SW. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* (Lond) 2000;408:1004–8.

Mansouri A, Zhang Q, Ridgway LD, Tian L, Claret FX. Cisplatin resistance in an ovarian carcinoma is associated with a defect in programmed cell death control through XIAP regulation. *Oncol Res* 2003;13:399–404.

McEleny KR, Watson RW, Coffey RN, O'Neill AJ, Fitzpatrick JM. Inhibitors of apoptosis proteins in prostate cancer cell lines. *Prostate* 2002;51:133–40.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Sca□ di C, Bretz JD, ZhangM, Gentz R, Mann M, Kreammer PH, Peter ME and Dixit VM. *Cell* 1996;85: 817-827.

Nachmias B, Ashhab Y, Ben-Yehuda D. The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer. *Semin Cancer Biol* 2004;14:231–43.

Nishihara H, Kizaka-Kondoh S, Insel PA, Eckmann L. Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. *Proc Natl Acad Sci USA* 2003;100:8921–6.

Rajcan-Separovic, E., P. Liston, C. Lefebvre, and R.G. Korneluk. Assignment of human inhibitor of apoptosis protein (IAP) genes xiap, hiap-1, and hiap-2 to chromosomes Xq25 and 11q22-q23 by fluorescence in situ hybridization. *Genomics* 1996;37: 404–406.

Rothe M, Pan M-G, Henzel WJ, Ayres TM, Goeddel DV. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* 1995;83:1243–52.

Roy, N., M.S. Mahadevan, M. McLean, G. Shutler, Z. Yaraghi, R. Farahani, S. Baird, Besner-Johnson A., Lefebvre C., Kang X., Salih M., Aubry H., Tamai K., Guan X., Ioannou P., Crawford T.O., Jong P.J. de, Surh L., Ikeda J.-E., Korneluk R.G.,

and MacKenzie A. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* 1995;80: 167–178.

Roy N, Deveraux QL, Takashashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IaP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* 1997;16: 6914–6925.

Saleh A, Srinivasula S, Acharya S, Fishel R, Alnemri E. Cytochrome c and dATPmediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* 1999;274:17941–5.

Sanna MG, da Silva Correia J, Ducrey O, Lee J, Nomoto K, Schrantz N, Deveraux QL, Ulevitch RJ. IAP suppression of apoptosis involves distinct mechanisms: the TAK1/JNK1 signaling cascade and caspase inhibition. *Mol Cell Biol* 2002;22:1754–66.

Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. *Cell* 1997; 91:443–6.

Savopoulos, J.W., Carter, P.S., Turconi, S., Pettman, G.R., Karran, E.H., Gray, C.W., Ward, R.V., Jenkins, O., and Creasy, C.L. Expression, purification, and functional analysis of the human serine protease HtrA2. *Protein Expr. Purif.* 2000;*19:* 227–234.

Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)–kappaB–regulated X-chromosome–linked iap gene expression protects endothelial cells from tumor necrosis factor alpha–induced apoptosis. *J Exp Med* 1998;188:211 - 6.

Stennicke HR, Ju¨rgensmeier JM, Shin H, Deveraux Q, Wolf BB, Yang X, Zhou Q, Ellerby HM, Ellerby LM, Bredesen D, Green DR, Reed JC, Froelich CJ, Salvesen GS. Pro-caspase-3 is a major physiologic target of caspase-8. *J Biol Chem* 1998;273:27084–90.

Sundqvist K. Liu Y. Nair J. Bartsch H. Arvidson K. Grafstrom RC. Cytotoxic and

genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells. *Cancer Research.* 1989 Oct ;1 49(19):5294-8,.

Suzuki Y, Nakabayashi Y, Takahashi R. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc Natl Acad Sci USA* 2001;98:8662–7.

Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, Reed JC. A single BIR domain of XIAP sufficient for inhibiting caspases. *J. Biol. Chem.* 1998;273: 7787– 7790.

Tang D, Lahti JM, Kidd VJ. Caspase-8 activation and bid cleavage contribute to MCF7 cellular execution in a caspase-3-dependent manner during staurosporinemediated apoptosis. *J Biol Chem* 2000;275:9303–7.

Tanimoto T, Tsuda H, Imazeki N,Ohno Y, Imoto I, Inazawa J, Matsubara O. Nuclear expression of cIAP-1, an apoptosis inhibiting protein,predicts lymph node metastasis and poor patient prognosisin head and neck squamous cell carcinomas, *Cancer Letters* 2005;224: 141–151

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins, *Cell* 2000;102 :43–53

Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998;281:1680 - 3.

Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000;288:874–7.

Young SS, Liston P, Xuan JY, McRoberts C, Lefebvre CA, Korneluk RG. Genomic organization of the physical map of the human inhibitors of apoptosis: HIAP1, HIAP2. *Mamm Genome.* 1999;10(1):44-8

Yuan J, Horvitz HR. The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development.* 1992;116(2):309-20 .

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997;90:405–13.

**Table 1.** The mean cIAP-1 labeling indices (LI) in normal oral mucosa (NOM), oral epithelial dysplasia (OED), and oral squamous cell carcinoma (OSCC) samples

| Groups | Mean cytoplasmic cIAP-1 LI ± SD (%) |
|---|---|
| NOM (n = 31) | 23 ± 22 |
| OED (n = 76) | 50 ± 25 |
| Mild (n = 23) | 40 ± 24 |
| Moderate (n = 34) | 45 ± 27 |
| Severe (n = 19) | 72 ± 22 |
| OSCC (n = 73) | 73 ± 17 |

A significant difference in the mean cytoplasmic cIAP-1 LI was found among NOM, OED, and OSCC samples ($P$ = 0.000). The following comparisons were statistically significant for the mean cytoplasmic cIAP-1 LI: NOM v.s. mild OED, $P$ = 0.009; NOM v.s moderate OED, severe OED, or OSCC, $P$ = 0.000; mild OED vs. severe OED or OSCC, $P$ = 0.000; and moderate OED vs. severe OED or OSCC, $P$ = 0.000.

**Table 2.** Correlation between cytoplasmic cIAP-1 labeling indices (LI) in OSCC samples and clinicopathological parameters of 73 OSCC patients

| | Mean cytoplasmic cIAP-1 LI ± SD (%) | *P*-value |
|---|---|---|
| Age | | 0.214 |
| < 50 (n = 32) | 76 ± 18 | |
| ≧ 50 (n = 41) | 71 ± 16 | |
| Gender | | 0.862 |
| Men (n = 63) | 73 ± 16 | |
| Women (n = 10) | 72 ± 22 | |
| Location | | 0.139 |
| Buccal mucosa (n = 41) | 75 ± 17 | |
| Other sites (n = 32) | 69 ± 17 | |
| T status | | 0.322 |
| T1 + T2 (n = 36) | 71 ± 15 | |
| T3 + T4 (n = 37) | 75 ± 19 | |
| N status | | **0.000** |
| N0 (n = 45) | 66 ± 17 | |
| N1 + N2 + N3 (n = 28) | 84 ± 10 | |
| Clinical staging | | **0.045** |
| Stage 1 + 2 (n = 28) | 68 ± 15 | |
| Stage 3 + 4 (n = 45) | 76 ± 17 | |
| Histology of SCC | | 0.518 |
| WD OSCC (n = 64) | 74 ± 18 | |
| MD OSCC (n = 9) | 70 ± 10 | |
| Recurrence | | 0.332 |
| With (n = 31) | 71 ± 20 | |
| Without (n = 42) | 75 ± 15 | |

Comparison between groups was performed by Student's *t*-test.

WD = well-differentiated, MD = moderately-differentiated

**Table 3** Correlation between cytoplasmic cIAP-1 labeling indices (LI) in OSCC samples and oral habits (OH) of OSCC patients

| | Mean cytoplasmic cIAP-1 LI ± SD (%) | *P*-value |
|---|---|---|
| Alcohol | | 0.239 |
| Drinkers (n = 50) | 75 ± 17 | |
| Non-drinkers (n =23) | 70 ± 16 | |
| Areca quid | | 0.061 |
| Chewers (n = 57) | 75 ± 15 | |
| Non-chewers (n = 16) | 66 ± 22 | |
| Cigarette | | 0.165 |
| Smokers (n = 59) | 74 ± 16 | |
| Non-smokers (n = 14) | 67 ± 20 | |
| Combination | | 0.193 |
| Patients with at least one OH (n = 64) | 74 ± 16 | |
| Patients without any OH (n = 9) | 66 ± 24 | |

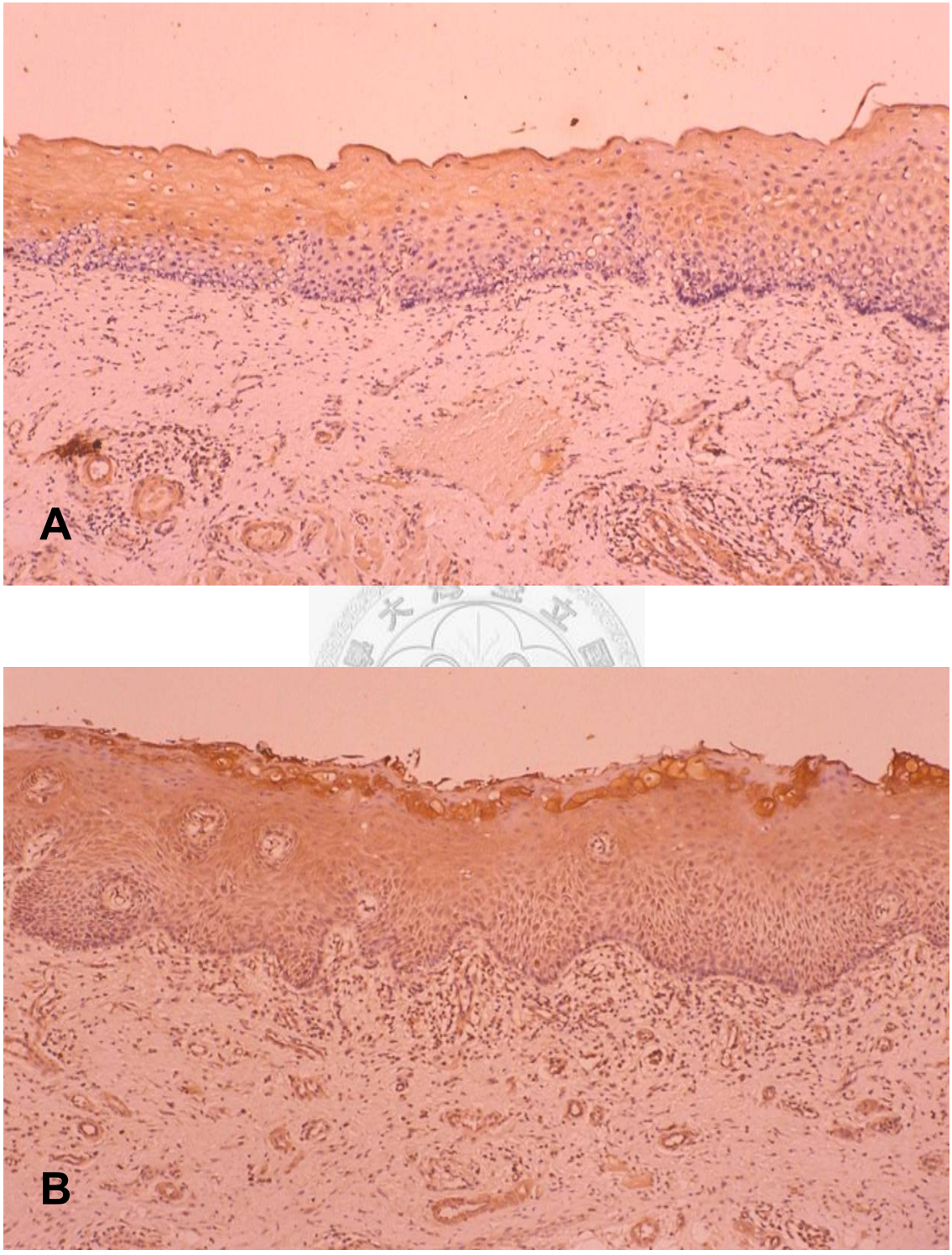
Comparison between groups was performed by Student's *t*-test

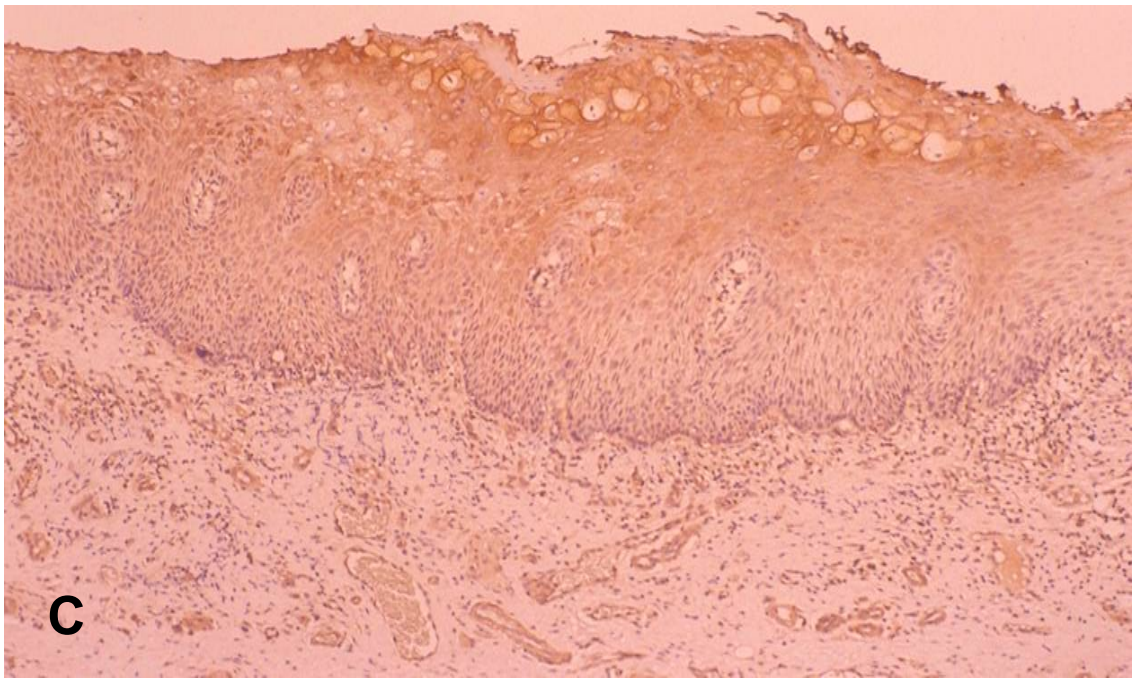
**Table 4.** Univariate and multivariate survival analyses of cIAP-1 LI and clinicopathological parameters in 73 patients with OSCC by Cox proportional hazard regression model

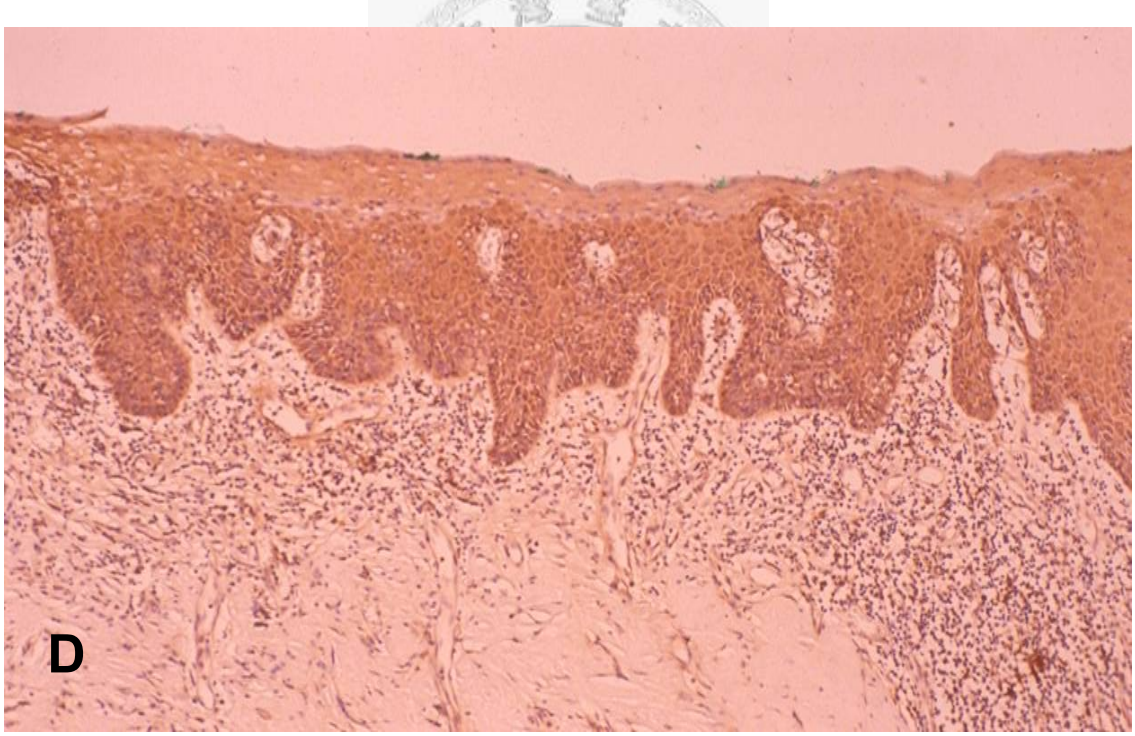
| Factor | Hazard ratio (95% CI) | *P*-value |
|---|---|---|
| **Univariate** | | |
| N status (N0 vs. N1 + N2 + N3) | 2.748 (1.267~5.957) | **0.0105** |
| Clinical stage (Stage 1 + 2 vs. stage 3 + 4) | 2.354 (0.992~5.587) | **0.0523** |
| cIAP-1 LI (LI ≤ 75% vs. LI > 75%) | 1.202 (0.561~2.577) | 0.6361 |
| **Multivariate** | | |
| cIAP-1 LI (LI ≤ 75% vs. LI > 75%) | 0.611 (0.242~1.539) | 0.2958 |

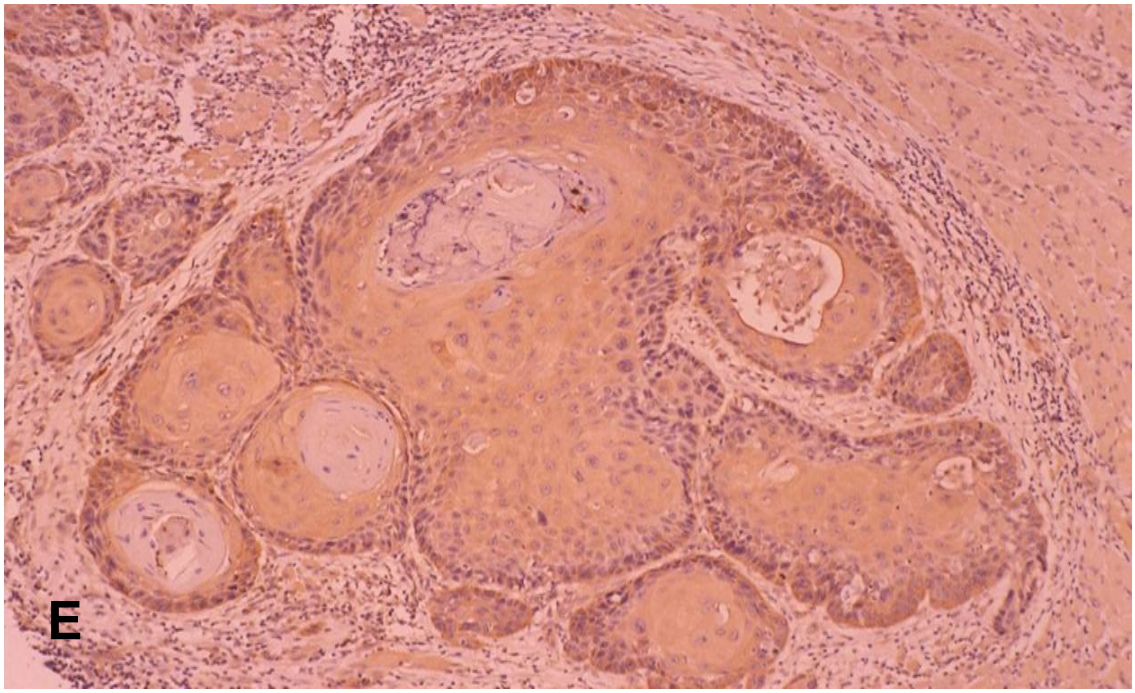
**Figure 1.** Immunohistochemical staining for cIAP-1. (A) Normal buccal mucosa showing cytoplasmic cIAP-1 staining in cells of the upper half layer of the epithelium. (B) Buccal mild epithelial dysplasia exhibiting cytoplasmic cIAP-1 staining mainly in cells of the upper half layer of the epithelium. (C) Buccal moderate epithelial dysplasia demonstrating cytoplasmic cIAP-1 staining mainly in cells of the upper two-thirds layer of the epithelium. (D) Buccal severe epithelial dysplasia showing cytoplasmic cIAP-1 staining in the whole layer epithelial cells. (E) Buccal well-differentiated squamous cell carcinoma exhibiting cytoplasmic cIAP-1 staining in nearly all cells of the cancer nests except those in the keratin pearls. (F) Buccal well-differentiated squamous cell carcinoma demonstrating denser cytoplasmic cIAP-1 staining in cancer cells near the invasion front than in cancer cells far away from the invasion front. (Original magnification; A, B, C, D and F, 25×, E, 50×)

**Fig 1**




C


D


E

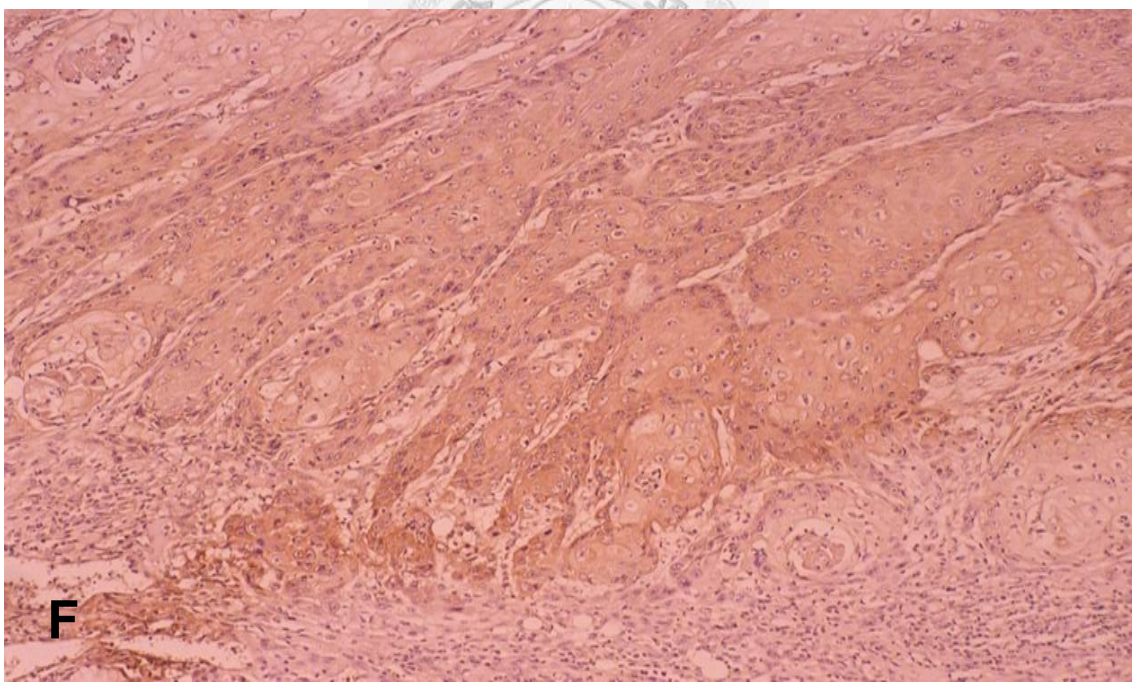
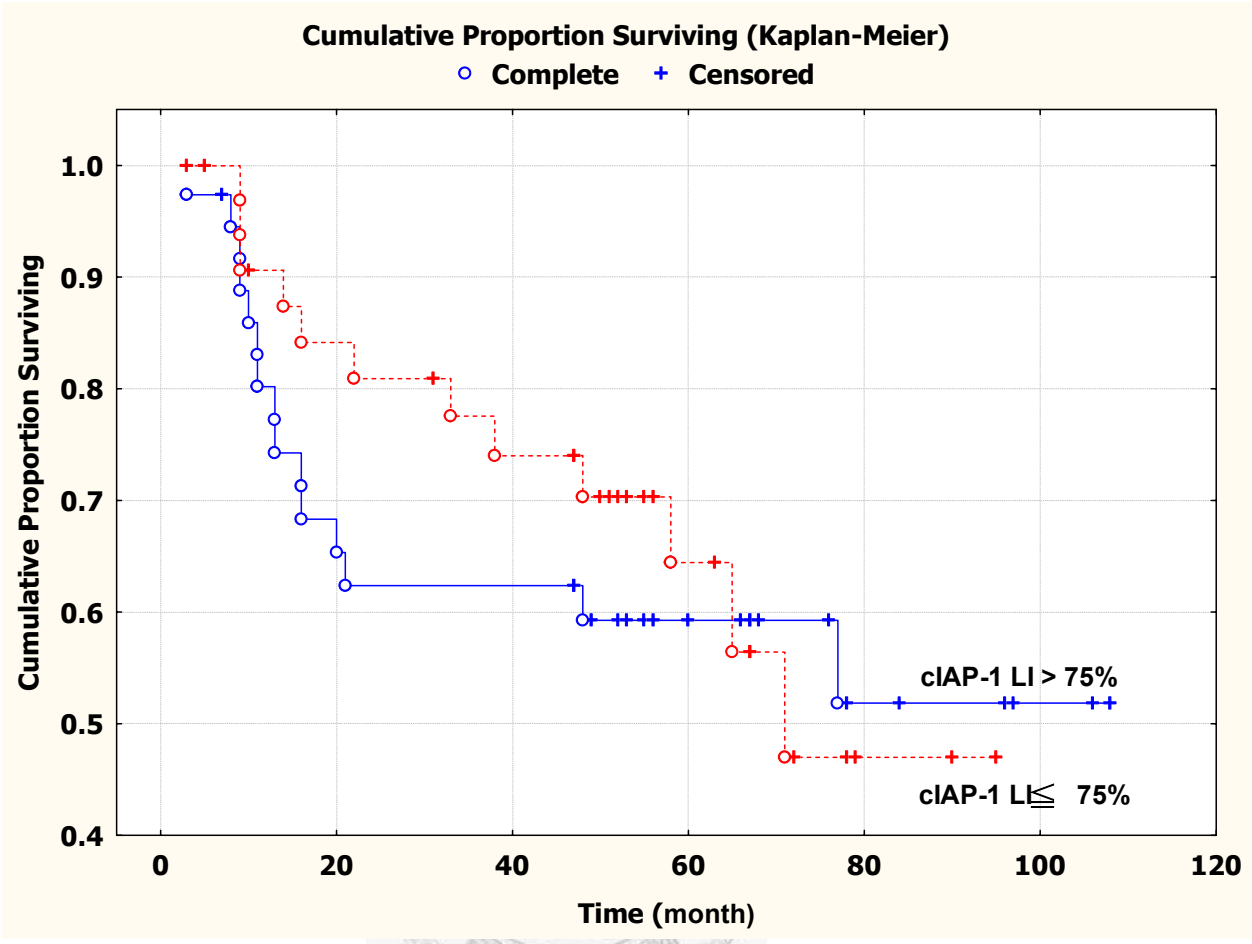

F

**Figure 2**. The correlation between cytoplasmic cIAP-1 LI in OSCCs and survival of OSCC patients was analyzed with Kaplan-Meier product-limit method. There is no significant difference between cytoplasmic cIAP-1 LI in OSCCs and survival of OSCC patients.

# Figure 2




Log-Rank Test, p = 0.63154