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博士論文

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Doctoral Dissertation

口腔癌前病變之光動力及冷凍治療

Photodynamic therapy and cryotherapy for oral precancerous lesions

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謝誌

陳之藩先生曾說：「要感謝的人太多了，就感謝天吧！」回顧這幾年研究所的生活，我想要感謝的人很多。除了感謝天之外，我還要寫下對幾個人的感謝之意。

首先我要感謝我的摯友蔡尚學先生，我們是從高二就認識的同學。我之所以從事醫療行業以及加入童軍運動，絕大部份是受到他的影響。他啓發我對醫學精神的實踐、對人文素養的追求、以及對周遭人事物的關懷，在生活上也給我許多資助。在我人格養成的重要階段，很幸運遇到像他這樣好的人，能在德業上互相勉勵。可惜人世無常，在一年多前的十一月凌晨，他竟無預警地撒手人寰而英年早逝，留下妻子與一名稚兒，令人悵然若失、無限感傷。孔老夫子說：「益者三友：友直，友諒，友多聞。」唐太宗在魏徵死後曾說：「以銅為鏡，可以正衣冠；以古為鏡，可以知興替；以人為鏡，可以明得失。朕嘗寶此三鏡，用防己過。今魏徵殂逝，遂亡一鏡矣。」尚學真可說是我這一生不可多得的益友、摯友。在他過世的一年後，回憶起他對我的恩惠，仍不覺熱淚盈眶，無限哀思。

其次我要感謝我的指導教授江俊斌老師。老師慈眉善目、和藹可親，總是不厭其煩地教導我。老師給我許多機會出國參加會議，拓展視野、增廣見聞，尤其是遠赴非洲三個國家，與當地的醫院及學校作學術交流，更是這輩子難得與難忘的經驗。老師並鼓勵我擔任牙醫系助教，培養我如何教導學生，同時讓我有穩定的收入可以專心念書。承蒙老師的厚愛與提攜，也擔任口腔病理學會秘書長，可以認識學會的前輩們，從中學習前輩的風範。老師也常鼓勵我多寫、多發表論文。他告訴我：「發表的論文是跟著你一輩子，以後要申請教職或到醫院申請主治醫師都很有幫助。」因此我至今有二十多篇的論文，完全得力於老師的鼓勵。老師指導我的方式非常自由開明，從不規定我那個時候一定要將那個實驗做完；當我沒有將事情做得很完善的時候，老師也從不生氣，也不曾念過我一句，只是輕輕說一聲「沒關係」，讓我感到很愧咎。老師有一點最令我佩服的是，每當我有事情請教老師的時候，不論他有多忙或是他正在處理什麼事情，老師一定會放下手邊的工作，很有耐心地解決我的問題。我自覺我個性內向、不夠聰明、不太會說話、不會舉一反三，有時呆頭呆腦又迷糊，一個疾病讀了十幾遍還是背不起來。牛頓（Sir Isaac Newton）在一六七六年給友人的信中寫道：「如果說我看得比別人更遠，那是因為我站在巨人的肩膀上。」（If I have seen farther than others, it is because I was standing on the shoulders of giants.）。如果我能在學術上有一點點微不足道的成就，無疑是因為我有「巨人」般的老師給我的教導、鼓勵與提攜。

我還要感謝我的女朋友珈融。認識她的時候，我已大學畢業，正是脫離「為賦新詞強說愁」的年紀；而珈融就讀大一，正值「少女情懷總是詩」的青春年華。從我們認識至今，大部份的時間都是分隔兩地，只有放假才能相聚。畢業之後我就忙於工作及研究所的課業，許多大學時候唐詩宋詞般的多愁善

感、寫詩寫散文寫日記的閒情逸致，都差不多銷磨殆盡，因此我和珈融之間幾乎沒有什麼浪漫的愛情生活。一千多個日子常常在平淡無奇與兩地相思之中度過。但她總能非常體貼地體諒我，不曾有過太多的怨言。有了她默默的、溫柔的陪伴與支持，我在研究所的生活才不致過於孤單。

最後我要感謝我的父親余清儹先生與母親柯嫊美女士。我從高中開始就離家在外地求學，從嘉義、台中、到台北，一路北上求學，至今已將屆十六年。這十六年來不常隨侍親側、略盡孝道，只能偶爾打電話回家請安。父親年近耳順之年，擔任公職三十多年，一個人獨自賺錢培養我家四個小孩，對於我的教育，始終讓我自由發展，鼓勵遠大於責備。我小時候身體虛弱，經常生病，父親總是不辭辛勞地帶我去看醫生；讀私立學校牙醫系要花許多錢買教科書、器械、繳房租，父親也不曾要我去打工貼補家用，只告訴我要好好讀書，不用擔心錢的事情。如今我年過而立之年，家未成、業未立，不曾賺得許多錢孝養父母，卻還在讀著永遠讀不完的書，心中著實有十二萬分的歉意。母親年過知天命之年，是個刻苦耐勞的家庭主婦，含辛茹苦地拉拔我們四個小孩長大。每當我打電話回家請安，母親總不忘叮嚀我要吃多一點、吃胖一點、吃東西的錢不用省、要多照顧自己、天氣冷要多穿一件衣服、不要太晚睡等等。母親對我的呵護與關心，時常讓我想起孟郊的《遊子吟》：「慈母手中線，遊子身上衣；臨行密密縫，意恐遲遲歸；誰言寸草心，報得三春暉。」父母的恩情真是一輩子也報答不完。席慕容的詩《鄉愁》裡有句話說：「故鄉的歌是一支清遠的笛，總在有月亮的晚上響起。」離鄉背井十多年，有時在夜闌人靜、萬籟俱寂的夜晚，想起在故鄉日漸蒼老的父母，卻無法時常承歡膝下，往往悲從中來，潸然淚下。只盼望能早日完成學業，奉養父母。

史懷哲（Albert Schweitzer）說他在二十一歲那年有個願望：「我決定三十歲以前讓自己繼續為學問和藝術而生活，這是有道理的，為的是三十歲以後有足夠的能力奉獻自己，直接服務人群。」於是他在三十歲那年放棄他最喜愛的工作及優渥收入的生活，進入醫學院研讀醫學。在三十八歲獲得醫學博士學位之後，便與他的夫人至非洲行醫長達五十二年。如今我已年過而立，正好開始為實現自己的理想而奮鬥。先哲朱柏廬先生在《治家格言》提到：「讀書志在聖賢」。讀書人應當要有宋朝先儒張橫渠先生「為天地立心，為生民立命，為往聖繼絕學，為萬世開太平」的使命感。孔老夫子在《孝經》的開宗明義章告訴我們：「立身行道，揚名於後世，以顯父母，孝之終也。」又說：「夫孝，始於事親，中於事君，終於立身。」而引用《詩經．大雅．文王篇》的一句話「無念爾祖，聿修厥德」勉勵我們要念念不忘祖先懿德，修養自己，以發揚祖先美好的德行。台灣總督府醫學校第二任校長高木友枝先生經常勉勵畢業生：「要做醫生之前，必須做成了人，沒有完成的人格，不能負醫生的責務。」自今而後，期許自己做一個術德兼備的牙醫師，恪遵聖賢教誨，努力實現自己的理想，以報答父母養育之恩與師長栽培之情。

國立台灣大學醫學院臨床牙醫學研究所
博士學位論文
口試委員會審定書

論文題目

口腔癌前病變之光動力及冷凍治療

Photodynamic therapy and cryotherapy for oral precancerous lesions

本論文係余權航君（學號 D94422004）在國立臺灣大學醫學院臨床牙醫學研究所完成之博士學位論文，於民國九十七年五月十三日承下列考試委員審查通過及口試及格，特此證明。

考　　試　　委　　員				

中華民國九十七年五月十三日

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I. 中文摘要

背景：口腔白斑（oral leukoplakia, OL）、紅白斑（oral erythroleukoplakia, OEL）及疣狀增生（oral verrucous hyperplasia, OVH）是三種常見的口腔癌前病變。本研究中，我們採用兩種保守性的治療方法，即以局部塗抹 5－氨基酮戊酸為導引之光動力療法（5-aminolevulinic acid-mediated photodynamic therapy, ALA-PDT）與棉棒冷凍療法（cotton-swab cryotherapy, CSC），來治療這些病變。此外，我們也利用免疫組織化學染色的方法，來評估細胞凋亡相關蛋白在接受 PDT 前活體切片組織中的表現量，是否可以作為預測局部 ALA-PDT 療效的生物標記。

方法：本研究收集 36 例 OVH、65 例 OL 及 20 例 OEL 病變，以局部 ALA-PDT 治療，每週治療一次，另 32 例 OL 病變，以相同的局部 ALA-PDT 方式治療，每週治療兩次。我們以卡方檢定（chi-square test）來比較任兩組之臨床療效差異。同時我們也評估那些臨床及病理參數，會影響局部 ALA-PDT 對 OVH 病變的療效。我們將病變對治療的反應分成三類：完全反應（complete response, CR）、部分反應（partial response, PR）以及無反應（no response, NR）。本研究利用對抗 Bak、Mcl-1、caspase-3、caspase-8、caspase-9、p53、p21 及 PCNA 等蛋白的抗體與免疫組織化學染色法，來觀察 18 例 OVH 與 40 例 OL 病變，在接受 PDT 前活體切片組織中這些蛋白的表現量。我們分別記錄這些蛋白在細胞質與細胞核的標記指數（labeling indices, LIs）及染色強度（staining intensity, SI），並利用統計方法來比較 CR 與 PR 或 NR 兩組之間，在表面角質層厚度與標記分數（labeling score, LS，定義為 LI × SI）平均值的差異。本研究同時也使用 CSC 方法治療 47 位病人共 60 例 OL 病變，患者每兩週治療一次，直到病變完全消除為止。

結果：所有 36 例 OVH 病變，於平均 3.8 次的局部 ALA-PDT 治療之後，皆得到 CR。65 例 OL 病變，以局部 ALA-PDT 每週治療一次，發現有 5 例為 CR，33

例為 PR，27 例為 NR。32 例 OL 病變，以相同的局部 ALA-PDT 方式，每週治療兩次，發現有 11 例達到 CR，21 例呈現 PR。另 20 例 OEL 病變，以局部 ALA-PDT 每週治療一次，發現有 17 例為 CR，3 例呈現 PR。比較各組間之療效發現，32 例 OL 病變以局部 ALA-PDT 每週治療兩次的療效，比 65 例 OL 病變以局部 ALA-PDT 每週治療一次的療效顯著較佳（$P < 0.001$）。另外，20 例 OEL 病變以局部 ALA-PDT 每週治療一次的療效，比 65 例 OL 病變以局部 ALA-PDT 每週治療一次的療效也顯著較佳（$P = 0.000$）。此外，當 OVH 病變的外形為腫塊、最大徑小於 1.5 公分、外觀為粉紅色、上皮細胞發生變異，以及表面角質層厚度小於或等於 40 微米時，比起其外形呈現斑狀或合併中間為腫塊但周圍為斑狀（$P = 0.000$）、最大徑大於或等於 1.5 公分（$P = 0.011$）、外觀為白色（$P = 0.000$）、上皮細胞無變異（$P = 0.043$）或表面角質層厚度大於 40 微米（$P = 0.003$）者，需明顯較少的治療次數就能達到 CR。以多變項統計分析顯示，OVH 病變的外觀（$P = 0.0069$），為可用來預測局部 ALA-PDT 療效的唯一獨立因子。免疫組織化學染色的結果顯示，CR 組別的 Bak 標記分數遠高於 PR 或 NR 組別（$P = 0.003$）。同時我們也發現 CR 組別的 Bak/Mcl-1 比值遠高於 PR 或 NR 組別（$P = 0.02$）。我們也觀察到 CR 組別的表面角質層厚度，遠低於 PR 或 NR 組別（$P = 0.0036$）。所有 60 例 OL 病變，在經過平均 6.3 次 CSC 治療之後，均呈現 CR。當 OL 病變位於舌以外的口腔黏膜上、面積小於 2 平方公分、上皮發生變異、或表面角質層厚度小於 55 微米時，其達到 CR 所需要的治療次數，明顯少於其位於舌（$P = 0.003$）、面積大於或等於 2 平方公分（$P = 0.024$）、上皮無變異（$P = 0.033$）、或表面角質層厚度大於或等於 55 微米（$P = 0.045$）者。以多變項統計分析顯示，OL 病變的位置（$P = 0.000176$）與面積（$P = 0.021280$）為影響 CSC 達到 CR 療效的獨立預測因子。

結論：局部 ALA-PDT 對治療 OVH 病變非常有效。當 OVH 病變最大徑小於或等於 3.1 公分，經由每週一次局部 ALA-PDT 治療，可在少於 7 次的療程時達到 CR。局部 ALA-PDT 對 OVH 病變的療效，取決於病變的外形、大小、顏色、

上皮是否變異，以及表面角質層的厚度。OVH 病變的外觀是影響 ALA-PDT 療效的唯一獨立因子。OL 病變以局部 ALA-PDT 每週治療兩次，比每週治療一次，有顯著較佳的療效。OEL 病變以局部 ALA-PDT 每週治療一次，比 OL 病變以局部 ALA-PDT 每週治療一次的療效顯著較佳。OVH 及 OL 病變在 PDT 之前的活體切片組織中，其 Bak 的表現量與 Bak/Mcl-1 表現量的比值，可當作預測 PDT 療效的生物標記。CSC 對 OL 病變而言是一種簡單、安全、方便與保守的療法。當 OL 病變的表面積介於 0.1 至 6.5（平均 1.8）平方公分之間，以平均少於 7 次的 CSC 療程，即可達到 CR。當 OL 病變位於舌以外的口腔黏膜上、上皮發生變異、以及具較薄的表面角質層厚度，達到 CR 所需要的治療次數，明顯少於病變位於舌頭、上皮無變異、或具較厚的表面角質層厚度者。

關鍵字：5－氨基酮戊酸、口腔癌前病變、光動力治療、免疫組織化學染色、冷凍治療

II. ABSTRACT

Background: Oral leukoplakia (OL), oral erythroleukoplakia (OEL), and oral verrucous hyperplasia (OVH) are three common oral precancerous lesions. In this study, we used two conservative treatment modalities, topical 5-aminolevulinic acid-mediated photodynamic therapy (ALA-PDT) and cotton-swab cryotherapy (CSC), for treatment of these lesions. In addition, we also used immunohistochemistry (IHC) to study whether the expression of apoptosis-associated proteins in oral precancerous lesions before PDT could be a biomarker to predict the treatment outcomes of topical ALA-PDT.

Methods: Thirty-six OVH lesions, 65 OL lesions, and 20 OEL lesions were treated with topical ALA-PDT once a week and 32 OL lesions were treated with the same topical ALA-PDT twice a week. Their clinical outcomes between 2 different groups were compared by chi-square test. We also assessed what clinicopathological parameters of OVH lesions could influence PDT treatment outcomes. Lesion response was characterized into three categories: complete response (CR), partial response (PR), and no response (NR). IHC was performed with antibodies against Bak, Mcl-1, caspase-3, caspase-8, caspase-9, p53, p21, or PCNA protein in 18 OVH and 40 OL biopsy specimens taken before PDT. Both the labeling indices (LIs) and staining intensity (SI) of cytoplasmic or nuclear staining by each antibody were recorded. The means of surface keratin thickness or labeling score (LS, defined as LI × SI) were statistically compared between the CR and PR or NR group. Sixty OL lesions from 47 patients were treated with CSC once 2 weeks until CR of the lesion.

Results: All 36 OVH lesions showed CR after an average of 3.8 treatments of topical ALA-PDT. The 65 OL lesions treated with topical ALA-PDT once a week showed CR in 5, PR in 33, and NR in 27. The 32 OL lesions treated with the same topical ALA-PDT twice a week demonstrated CR in 11 and PR in 21. The 32 OL lesions treated

twice a week had a significantly better clinical outcome than the 65 OL lesions treated once a week ($P < 0.001$). The 20 OEL lesions treated with topical ALA-PDT once a week showed CR in 17 and PR in 3. The 20 OEL lesions treated once a week had a significantly better clinical outcome than the 65 OL lesions treated once a week ($P = 0.000$). In addition, OVH lesions with the clinical appearance of a mass, with the greatest diameter < 1.5 cm, with the pink color, with epithelial dysplasia, or with the surface keratin layer ≦ 40 μm needed significantly less mean treatment numbers of PDT to achieve a CR than OVH lesions with the clinical appearance of a plaque or a combination type of peripheral plaque and central mass ($P = 0.000$), with the greatest diameter ≧ 1.5 cm ($P = 0.011$), with the white color ($P = 0.000$), without epithelial dysplasia ($P = 0.043$), or with the surface keratin layer > 40 μm ($P = 0.003$), respectively. Multivariate analysis showed that only the clinical appearance of OVH lesions was the independent factor ($P = 0.0069$) to predict the PDT treatment outcome. IHC results revealed that the Bak LS was significant higher in the CR group than in the PR or NR groups ($P = 0.003$). A significant difference in the Bak/Mcl-1 LS ratio was also found between the CR and PR or NR groups ($P = 0.02$). We also showed a significant difference in the surface keratin thickness between the CR and PR or NR groups ($P = 0.036$). All 60 OL lesions treated with CSC showed CR after an average of 6.3 treatments. OL lesions on the oral mucosal sites other than the tongue, < 2 cm^2, with epithelial dysplasia, or with the surface keratin thickness < 55 μm needed significantly less treatment number of CSC to achieve a CR than OL lesions on the tongue ($P = 0.003$), ≧ 2 cm^2 ($P = 0.024$), without epithelial dysplasia ($P = 0.033$), or with the surface keratin thickness ≧ 55 μm ($P = 0.045$), respectively. Multivariate analyses showed that only the location ($P = 0.000176$) and area ($P = 0.021280$) of OL lesions were independent factors to influence the treatment number of cryotherapy to achieve a CR.

Conclusion: Topical ALA-PDT is a very effective treatment modality for OVH lesions. For OVH lesions less than or equal to 3.1 cm in greatest diameter, CR of the lesions can be achieved by less than 7 treatments of topical ALA-PDT once a week. The PDT treatment outcome for OVH depends on the clinical appearance, size, color, epithelial dysplasia, and surface keratin thickness of the lesion. The clinical appearance of OVH lesions is the only independent factor affecting the PDT treatment outcome. OL lesions treated with topical ALA-PDT twice a week have a significantly better clinical outcome than OL lesions treated with the same PDT protocol once a week. OEL lesions treated with topical ALA-PDT once a week have a significantly better clinical outcome than OL lesions treated with the same PDT protocol once a week. The Bak LS and the Bak/Mcl-1 LS ratio in tissue sections of OVH and OL lesions before PDT can be used as biomarkers to predict the PDT treatment outcomes. CSC technique is a simple, safe, easy, and conservative treatment modality for OL lesions. For OL lesions with the surface area ranging from 0.1 to 6.5 (mean, 1.8) cm^2, CR of the lesion can be achieved by less than 7 CSC treatments in average. OL lesions on oral mucosal sites other than the tongue, with dysplasia, and with thinner surface keratin layer needed significantly less treatment number of CSC to achieve a CR than OL lesions on the tongue, without dysplasia, and with thicker surface keratin layer, respectively.

KEYWORDS: 5-aminolevulinic acid, oral premalignant lesion, photodynamic therapy, immunohistochemistry, cryotherapy

III. INTRODUCTION

Oral cancer is the fifth most common cancer in the world (Lingen et al. 2001). The worldwide annual incidence of oral cancers was estimated to be 274,000, accounting for 2.5% of all malignancies in both sexes in 2002 (Parkin et al. 2002). Oral cancer occurs with an annual incidence of approximately 29,370 cases in the United States (Jemal et al. 2005). In Taiwan, oral cancers rank as the sixth most prevalent cancer in both sexes and account for the fourth most common cancer in males in 2006 (Taiwan area main causes of death 2006). The annual incidence was about 4,000 cases. The main etiologies that cause oral squamous cell carcinoma (OSCC) in Taiwan are areca quid (AQ) chewing, cigarette smoking, and alcohol consumption. There are two million people who habitually chew AQs (Ko et al. 1995) and 15.9% males over 18 years old have AQ chewing habit (Cancer registry annual report, ROC 2006); approximately 80% of all oral cancer deaths are associated with this habit (Kwan 1976).

Although various treatment modalities including radical surgical excision, chemotherapy and radiotherapy, separately or in combination, have been used for treatment of oral cancers, the survival rate for oral cancer patients in Taiwan remains low. The 5-year survival rate is 72% for those with stage I, 39% for those with stage II, 27% for those with stage III, and 12% for those with stage IV cancers (Chen et al. 1999). The low 5-year survival rate in patients with advanced oral cancers suggests the importance of early detection and treatment of oral cancers. In addition, one of the best strategies to prevent oral cancers is to identify the oral cancers at their precancerous stages and eliminate them to prevent their further transformation into oral cancers.

Oral leukoplakia (OL), oral erythroleukoplakia (OEL), and oral verrucous hyperplasia (OVH) are three common oral precancerous lesions that may transform

into an OSCC or an oral verrucous carcinoma (OVC). The malignant transformation rates of oral precancerous lesions are reported to be 1-7% for homogenous thick OL, 4-15% for granular or verruciform OL, 18-47% (28% in average) for OEL, 4-11% for moderate dysplasia, and 20-35% for severe dysplasia (Neville et al. 2002). Follow-up studies showed that OL lesions develop malignant transformation into OSCCs in an average time of 5-8 years (Silverman et al. 1984; Hogewind et al. 1989). A recent hospital-based case-control study demonstrated that the average malignant transformation times of OL lesions range from 7.2 years for a subject who chews AQ, smokes and drinks to 22.4 years for a subject without any risk factors (Shiu and Chen 2004). The high malignant transformation rates of OL and OEL lesions also highlight the importance of early detection and treatment of oral precancers. Traditionally, OL, OEL and OVH lesions can be eradicated by surgical excision that always leads to scar formation for a large lesion. Photodynamic therapy (PDT) is another effective treatment option for human premalignant and malignant lesions because it is non-invasive, is well tolerated by patients, can be used repeatedly without cumulative side effects, and results in little scar formation (Dolmans et al. 2003). Studies have demonstrated that topical 5-aminolevulinic acid-mediated PDT (ALA-PDT) is also an effective treatment modality for OL (Kubler et al. 1998; Sieron et al. 2001; Sieron et al. 2003) lesions.

PDT involves two individually non-toxic components, light and photosensitizer, that work together to induce cellular and tissue destruction in an oxygen-dependent manner. This technique is based on the administration of an exogenous photosensitizer to render tumor tissue sensitive to light of a specific wavelength. The photosensitizers are normally inert and have a selective affinity to tumor tissues. When a photosensitizer in tissues is activated by a light of specific wavelength, it transfers energy from light to molecular oxygen, resulting in generation of reactive

oxygen species (ROS) (Dolmans et al. 2003). There are three main mechanisms by which PDT mediates tumor destruction. Firstly, the ROS can kill tumor cells directly. Secondly, PDT can damage the tumor-associated vasculature, leading to thrombus formation and subsequent tumor infarction. Thirdly, PDT can also activate an immune response against tumor cells (Dolmans et al. 2003).

5-Aminolevulinic acid (ALA) itself is not a photosensitizer but serves as the biological precursor of the photosensitizer, protoporphyrin IX (PpIX), in the heme biosynthesis pathway. There are two rate-limiting steps in this pathway. One is the first step of forming ALA from glycine and succinyl CoA, which is regulated by heme via a negative feedback mechanism. The other rate-limiting step is the conversion of PpIX to heme, and this process is controlled by ferrochelatase, which adds a ferrous iron to PpIX to form the heme. Exogenous ALA administration short-circuits the first step of porphyrin synthesis and subsequently leads to the accumulation of PpIX in the tissue. Furthermore, PpIX accumulation could be the result of a decreased conversion of PpIX to heme in tumor cells as a result of a decreased ferrochelatase activity (Kennedy et al. 1990; Kennedy et al. 1996).

Cutaneous photosensitivity is a common adverse effect in clinical practice of PDT. Compared to Photofrin®-mediated PDT, ALA-PDT has been shown to have less cutaneous photosensitivity (Kennedy et al. 1990; Kennedy et al. 1996). ALA can be rapidly cleared from the tissues and the body within 48 hrs. Thus, patients after ALA-PDT treatment have no problem of prolonged skin photosensitivity.

PDT with systemically or topically administered ALA can be used for the treatment of human cancers from the esophagus (Gossner et al. 1998), urinary bladder (Berger et al. 2003), skin (Gupta and Ryder 2003; Gold and Goldman 2004), and oral cavity (Grant et al. 1993a; Fan et al. 1996) as well as for the treatment of human precancerous lesions from the esophagus (Barr et al. 1996; Gossner et al. 1998;

Ackroyd et al. 2000), uterine cervix (Bodner et al. 2003), urinary bladder (Waidelich et al. 2001), skin (Gold and Goldman 2004), and oral cavity (Fan et al. 1996; Kubler et al. 1998; Sieron et al. 2001; Sieron et al. 2003) with relatively promising clinical outcomes.

There are two topical ALA-related photosensitizers that are available in the market for the treatment of cutaneous premalignant lesions. Levulan® Kerastick™ (DUSA Pharmaceuticals, Wilmington, MA, USA) is an ALA preparation that has already been approved by the Food and Drug Administration (FDA), USA. Metvix® (PhotoCure ASA, Norway) is mainly a methyl ALA preparation that has already been approved by the European Agency for the Evaluation of Medicinal Products (EMEA). For lesions of nonhyperkeratotic actinic keratoses of the face and scalp, a response rate of 70-100% can be achieved by treatment with PDT after topical application of these two ALA-related agents (Gold and Goldman 2004; Gupta and Ryder 2003). ALA-PDT is a noninvasive treatment modality that produces excellent cosmetic results and can be applied repeatedly without cumulative toxicity (Peng et al. 1997). Previous studies by Leunig and his colleagues (Leunig et al. 1996; Leunig et al. 2000; Leunig et al. 2001) demonstrated that topically applied ALA can be selective for oral premalignant and malignant tissues. In addition, ALA-induced PpIX has good fluorescent properties; therefore, the level of PpIX in tissues can be monitored by fluorescence spectroscopy, and PDT can be carried out at the time point when the PpIX reaches its maximal concentration in tissues.

In this study, we reported and compared the clinical outcomes of 36 OVH lesions, 65 OL lesions, and 20 OEL lesions treated with topical ALA-PDT once a week and of 32 OL lesions treated with the same topical ALA-PDT twice a week. We tried to test the efficacy of this protocol on OVH lesions, to test whether OL lesions treated with topical ALA-PDT twice a week had a significantly better clinical outcome than OL

lesions treated once a week by the same treatment protocol, and to assess whether OEL lesions treated with topical ALA-PDT once a week had a significantly better clinical outcome than OL lesions treated once a week by the same treatment protocol. We also assessed what clinicopathological parameters of 36 OVH lesions could influence PDT treatment outcomes. In this study, a light-emitting diode (LED) light device was used because it is a simpler, smaller, lighter, cheaper, and more portable light source than the laser machine, can induce comparable PDT effect as the laser machine, and has the high potential to replace the laser machine in clinical use for PDT in the near future. In addition, we also used immunohistochemistry (IHC) to study whether the expression of apoptosis-associated proteins in oral precancerous lesions before PDT could be a biomarker to predict the treatment outcomes of topical ALA-PDT.

Cryotherapy is also an effective treatment modality for OL lesions (Emmings et al. 1967; Miller 1969; Sako et al. 1972; Chapin and Burkes 1973; Bekke and Baart 1979; Gongloff et al. 1980; Gongloff and Gage 1983; Al-Drouby 1983; Yeh 2000). It is a method of local destruction of lesional tissue by freezing *in situ* (Gongloff et al. 1980). Cryotherapy is carried out in either an “open” or a “closed” system (Leopard 1975). The open-system cryotherapy involves the direct application of cryogen to the lesion with a cotton swab (Yeh 2000) or by an open spray (Leopard 1975). The closed-system cryotherapy offers a greater degree of control with more complex and delicate apparatus (Leopard 1975; Yeh 2000). The advantages of cryotherapy include bloodless treatment, very low incidence of secondary infection, and a relative lack of scarring and pain (Yeh 2000). Studies have shown the utility of cryotherapy to treat various oral lesions (Emmings et al. 1967; Gage 1969; Miller 1969; Hurt et al. 1972; Sako et al. 1972; Chapin and Burkes 1973; Leopard and Poswillo 1974; Weaver and Smith 1974; Leopard 1975; Chapin 1976; Bekke and Baart 1979; Reade 1979;

Gongloff et al. 1980; Tal et al. 1982; Gongloff and Gage 1983; Al-Drouby 1983; Loitz and O'Leary 1986; Tal and Gorsky 1987; Tal 1992; Toida et al. 1993; Ishida and Ramos-e-Silva 1998; Yeh 1998; Yeh 2000a; Yeh 2000b; Yeh 2003) including OL lesions (Emmings et al. 1967; Miller 1969; Sako et al. 1972; Chapin and Burkes 1973; Bekke and Baart 1979; Gongloff et al. 1980; Gongloff and Gage 1983; Al-Drouby 1983; Yeh 2000a).

Cotton-swab cryotherapy (CSC) technique, which was performed by direct application of liquid nitrogen to the lesion with a cotton swab, was first described by Toida et al (1993). It is a simple, easy method that does not require sophisticated skill and equipments (Toida et al. 1993). Nevertheless, there were few studies using CSC technique for treating oral lesions (Toida et al. 1993; Yeh 1998; Yeh 2000a; Yeh 2000b; Yeh 2003), and only one study used CSC technique for treating OL lesions (Yeh 2000a). In this study, we used the CSC technique to treat 60 OL lesions from 47 patients. Our main aim was to test the efficacy of cryotherapy on OL lesions. Furthermore, the relationship between the treatment number of cryotherapy to achieve a complete regression (CR) and clinicopathological parameters of 60 OL lesions was assessed by univariate and multivariate analyses.

IV. REVIEW OF LITERATURE

A. Oral precancerous lesions

Oral cancers account for 2% of all cancers in Western populations (Parkin et al. 1993), but are the most common forms of cancer in some Southeast Asian countries where the habit of AQ chewing is very popular (W.H.O. meeting report 1984). Among all oral malignancies, the OSCC was the most common type, representing more than 95% of all oral cancers (Muir and Weiland 1995). In Taiwan, oral cancer ranks as the fourth most common cancer in males in year 2005 and causes annual death of about 2,000 in males. The 5-year survival rate of oral cancers in males was 49% (Cancer registry annual report, ROC 2008). The deaths from oral cancers were 2,202 in year 2006, accounting for 5.8% (2,202 in 37,998) of deaths from all malignancies. Oral cancers rank as the sixth most prevalent cancer in both sexes and account for the fourth most common cancer in males in 2006 (Taiwan area main causes of death 2006). The mortality rate for oral cancer patients in both sexes and in males was 8.1% and 15.2%, respectively. The main etiologies that cause OSCCs in Taiwan are AQ chewing, cigarette smoking, and alcohol consumption. There are two million people who habitually chew AQs (Ko et al. 1995) and 15.9% males over 18 years old have AQ chewing habit (Cancer registry annual report, ROC 2006); approximately 80% of all oral cancer deaths are associated with this habit (Kwan 1976).

OL, OEL, and OVH are three common oral precancerous lesions. There are several terms to denote precancerous lesions of the oral mucosa such as “precancer”, “precursor lesion”, “premalignant lesion”, “intra-epithelial neoplasia”, and “potentially malignant lesion” (Warnakulasuriya et al. 2007). The oral precancerous lesion is defined as “a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart” (Alexll 1996) and was described under the term “epithelial precursor lesions” in the latest WHO monograph on Head

and Neck Tumors (Barnes et al. 2005a). Recently, an international working group recommend the term “potentially malignant disorder”, as it conveys that not all lesions and conditions described under this term may transform to cancers, rather that there is a family of morphological alterations amongst which some may have an increased potential for malignant transformation (Warnakulasuriya et al. 2007). Although the terms are different, all of them can be used to describe clinical lesions that may have a potential to become cancers.

OL is defined as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion (Axell 1996). Therefore, leukoplakia is a clinical term used when any other white oral lesion has been excluded (Lodi 2006). The recent international working group on oral precancer proposed definitions of OL as “recognized white plaques of questionable risk having excluded other known diseases or disorders that carry no increased risk for cancer (Warnakulasuriya et al. 2007). They concluded that a provisional diagnosis of OL is made when a predominantly white lesion at clinical examination cannot be clearly diagnosed as any other disease or disorder of the oral mucosa. A biopsy is mandatory. A definitive diagnosis is made when any etiological cause other than tobacco/areca nut use has been excluded and histopathology has not confirmed any other specific disorder (Warnakulasuriya et al. 2007). The prevalence rate of OL varies among countries. A recent systematic review revealed that the prevalence of leukoplakia is between 1.49% and 4.27%, and the global prevalence is 2.6% (Petti 2003). In Taiwan, oral carcinoma and dysplasia are found in 12.9% and 45.6% of biopsy OL lesions, respectively (Lee et al. 2006). Clinically, leukoplakia can be classified into two groups: (i) homogeneous leukoplakia, a lesion of uniform flat appearance that may have deep fissures and include thin and thick leukoplakia; (ii) non-homogeneous leukoplakia, a lesion that develop increased surface irregularities and may include granular, nodular,

verruciform leukoplakia and erythroleukoplakia (Lodi 2006). However, the histopathological feature of these two forms of leukoplakia is quite variable from merely ortho- or para-keratosis to various degrees of epithelial dysplasia or even early invasive carcinoma. The WHO histopathological classification of oral precancerous lesions (oral epithelial precursor lesions) are categorized as: (i) squamous cell hyperplasia, (ii) mild dysplasia, (iii) moderate dysplasia, (iv) severe dysplasia, and (v) carcinoma *in situ* (Barnes et al. 2005a).

Squamous cell hyperplasia describes increased cell numbers that may be in the spinous layer (acanthosis) and/or in the basal/parabasal cell layers (progenitor compartment), termed basal cell hyperplasia. The architecture shows regular stratification without cellular atypia. The term "dysplasia" was applied when architectural disturbance is accompanied by cytologic atypia. Dysplasia is a spectrum and no criteria exist to precisely divide this spectrum into mild, moderate and severe categories. In general architectural disturbance limited to the lower third of the epithelium accompanied by cytologic atypia defined the minimum criteria of dysplasia. Moderate dysplasia was defined when architectural disturbance extending into the middle third of the epithelium. However, consideration of the degree of cytologic atypia may require upgrading. The recognition of severe dysplasia starts with greater than two thirds of the epithelium showing architectural disturbance with associated cytologic atypia. Moderate dysplasia with sufficient cytologic atypia should be upgraded from moderate to severe dysplasia. The diagnosis of carcinoma *in situ* lies on the full thickness or almost full thickness architectural abnormalities in the viable cellular layers accompanied by pronounced cytologic atypia. Atypical mitotic figures and abnormal superficial mitoses are commonly seen in carcinoma *in situ* (Barnes et al. 2005a).

Non-homogeneous leukoplakias carry a higher degree of risk of transformation

when compared with the homogeneous variants (Lodi et al. 2006). It has reported that the global transformation rate for OL is 1.36% per year (Petti 2003). In Taiwan, the relative risks for the presence of malignancy in leukoplakias with non-homogeneous appearance were 28.13-fold when compared with lesions having homogeneous surface (Lee et al. 2006). In addition, non-homogeneous leukoplakia on tongue/floor of mouth has a 43.10-fold higher risk compared to homogeneous lesions located on buccal mucosa or other sites (Lee et al. 2006). The malignant transformation rates of oral premalignant lesions are reported to be 1-7% for homogenous thick OL, 4-15% for granular or verruciform OL, 18-47% (28% in average) for OEL, 4-11% for moderate dysplasia, and 20-35% for severe dysplasia (Neville et al. 2002). Follow-up studies showed that OL lesions develop malignant transformation into OSCCs in an average time of 5-8 years (Silverman et al. 1984; Hogewind et al. 1989). A hospital-based case-control study demonstrated that the average malignant transformation times of OL lesions range from 7.2 years for a subject who chews AQ, smokes and drinks to 22.4 years for a subject without any risk factors (Shiu and Chen 2004). Recently, Hsue et al. (2007) reported a 10-year follow-up study of the malignant transformation in 1,458 patients with potentially malignant oral mucosal disorders based on the samples from a Taiwanese hospital. They found that patients with epithelial dysplasia and submucous fibrosis have a 5.4% malignant transformation rate in an average time of 40.0 months. In addition, those with epithelial dysplasia and hyperkeratosis or epithelial hyperplasia have a malignant transformation rate of 4.65% in a mean time of 28.2 months (Hsue et al. 2007).

OVH may transform into an OVC or an OSCC. OVH was firstly described in detail by Shear and Pindborg (1980). Clinically, OVH lesions have papillary or pointed surface projections, varying keratin thickness, and broad, blunted epithelial ridges (Neville 2002). Histopathologically, OVH shows epithelial hyperplasia with

parakeratosis or hyperkeratosis and verrucous surface. There is no invasion of the hyperplastic epithelium into the lamina propria as compared to adjacent normal oral mucosal epithelium. Sometimes it may be difficult to differentiate an OVH from an OVC lesion. The characteristic histological feature distinguishing an OVC from an OVH lesion is that in addition to the surface verrucous projections there are also deep extensions of the OVC into the underlying connective tissue (Shear and Pindborg 1980). OVH is probably an irreversible precursor stage of OVC (Batsakis et al. 1999). In Taiwan, the malignant transformation rate of OVH is 3.09% in an average time of 54.6 months (Hsue et al. 2007).

Oral erythroplakia (OE) is a much less common precancerous lesion than OL (Reichart and Philipsen 2005). The prevalence of OE has a range between 0.02% and 0.83% (Reichart and Philipsen 2005). OE is defined as "a fiery red patch that cannot be characterized clinically or pathologically as any other definable lesion" (Pindborg et al. 1997). The clinical features of OE may present as a smooth, granular or nodular appearance. Often there is a well-defined margin adjacent to mucosa of normal appearance (Pindborg et al. 1997). OE mainly occurs in the middle-aged and the elderly and has a male predilection (Scully 2004). The soft palate, the floor of the mouth, and the buccal mucosa are most commonly affected sites by OE (Scully 2004). The typical lesion of OE is less than 1.5 cm in diameter and half of the OE lesions are less than 1 cm, but lesions larger than 4 cm have been observed (Bouquot and Ephros 1995). The etiology of OE reveals a strong association with tobacco consumption and alcohol use (Reichart and Philipsen 2005). Histopathologically, 51% of OE lesions were diagnosed as invasive carcinoma, 40% as carcinoma *in situ* or severe dysplasia, and 9% as mild to moderate dysplasia (Shafer and Waldron 1975). Therefore, OE has the highest risk of malignant transformation compared to all other oral mucosal lesions at risk for transformation (Reichart and Philipsen 2005).

Proliferative verrucous leukoplakia (PVL) is a rare but distinctive high-risk clinical form of oral precancerous lesions (Barnes et al. 2005b). Up to 2007, there have been 137 patients with PVL reported (Cabay et al. 2007). PVL is defined by its progressive clinical course, changing from a benign to a premalignant and further to a malignant lesion (Cabay et al. 2007). Silverman et al. (1984) documented a high rate of malignant transformation in a subset of patients with verrucous leukoplakia. In 1985, Hansen et al. (1985) further characterized this type of lesion and introduced the term PVL. PVL develops initially as focal clinical hyperkeratosis (leukoplakia) that progressively becomes a wide multifocal disease with gross exophytic features (Batsakis et al. 1999). There is a female predilection with a female-to-male ratio of 4:1 (Cabay et al. 2007), but is probably no racial preference (Ghazali et al. 2003). The average age at diagnosis is 62 years. The most common site for PVL is the buccal mucosa in women and the tongue in men (Barnes et al. 2005b). The clinical feature of PVL is variable and has a progressive clinical presentation. Nevertheless, there are no criteria that dictate how extensive the leukoplakic changes should be or how many or which oral subsites should be involved in order to qualify for the diagnosis of PVL to date (van der Waal and Reichart, 2007). PVL most often manifests as a flat white keratotic lesion with a grainy or verrucous surface. It may present as a single distinct lesion or, more often, as scattered multifocal growths involving several oral mucosal sites. As the lesions progress, they may exhibit horizontal and vertical growth, eventually taking on a more exophytic granular or verruciform appearance. PVL may progress to multiple oral foci of OVC or OSCC over time in spite of numerous treatment interventions (Cabay et al. 2007). PVL exhibits progressive histopathological features that may be observed in a single biopsy, multiple biopsies taken from a patient at the same time, or serial biopsies taken over time. Frequently, a pathological diagnosis of benign cellular or minimal dysplastic changes is made,

while the clinically aggressive behavior of the lesion is more consistent with a diagnosis of carcinoma (Cabay et al. 2007). Approximately 74% of the PVL lesions progress to oral carcinomas (Cabay et al. 2007).

B. Photodynamic Therapy

(1) History

Light is one of the God's gracious gifts and is essential for the human life. Thomas Alva Edison, who is the greatest inventor of the 19th century and the early 20th, lighten up the modern life of the human beings by his great invention, the incandescent light bulb. Now the light is going to save the human life by means of PDT for treating various diseases.

The application of light as therapy was for more than three thousand years (Daniell and Hill 1991; Ackroyd et al. 2001). Ancient Egyptian, Indian and Chinese civilizations had known to use light to treat various diseases (Spikes 1985). The beginning of the modern light therapy, the "phototherapy", that use of light for treating diseases was developed by Dane Niels Finsen (Dolmans et al. 2003; Moan and Peng 2003). He found that red-light exposure prevents the formation and discharge of smallpox pustules and can be used to treat this disease. He also used ultraviolet light from the sun to treat cutaneous tuberculosis (Finsen 1901). He was awarded a Nobel Prize for his discoveries and is acknowledged as the founder of modern phototherapy.

The combination of light and certain chemicals that could induce cell death was observed more than 100 years ago. Oscar Raab, a German medical student, reported that certain wavelengths are lethal to infusoria in the presence of acridine in 1900 (Raab 1900; Dolmans et al. 2003). In the same year, a neurologist in France named J. Prime found that epilepsy patients who are treated with oral eosin develop dermatitis

in sun-exposed areas (Prime 1900; Dolmans et al. 2003). Later, Hermann von Tappeiner and A. Jesionek treated skin tumors with topically applied eosin and white light in 1903 (von Tappeiner and Jesionek 1903; Dolmans et al. 2003) and later introduced the term “photodynamic action” (“photodynamische wirkung”) in 1904 (von Tappeiner and Jodlbauer 1904; Moan and Peng 2003).

Experiments to test combinations of reagents and light lead to modern PDT (Dolmans et al. 2003). PDT can be defined as the administration of a nontoxic drug or dye known as a photosensitizer either systemically, locally, or topically to a patient bearing a lesion (frequently but not always cancer), followed after some time by the illumination of the lesion with visible light (usually long wavelength red light), which, in the presence of oxygen, leads to the generation of cytotoxic species and consequently to cell death and tissue destruction (Castano et al. 2004).

The most extensively studied photosensitizers so far are porphyrins, which were identified in the mid-nineteenth century. Hausmann performed the first studies with these agents (Hausmann 1911; Dolmans et al. 2003). In 1913, the German scientist Friedrich Meyer-Betz was the first to treat humans with porphyrins (Meyer-Betz 1913; Dolmans et al. 2003). He injected 200 mg hematoporphyrin into himself and became extremely photosensitive for more than two months. In the 1960s, Lipson and colleagues initiated the modern era of PDT at the Mayo Clinic (Lipson and Baldes 1960; Lipson et al. 1961). These studies involved a compound that was developed by Schwartz called “hematoporphyrin derivative” (HPD) (Schwartz et al. 1955). The development of PDT to clinical acceptance must be thanks to the pioneering work of Dougherty and his colleagues at the Roswell Park Memorial Cancer Institute in Buffalo, USA, in the 1970s and 1980s (Moan and Peng 2003). In 1976, Kelly and Snell (1976) initiated the first human trials with HPD to treat patients with bladder cancer. Dougherty et al. (1978) carried out the second study with HPD-PDT to treat

25 patients with a total of 113 primary or secondary skin tumors. Following the preliminary successes in treating bladder and skin tumors, Hayata et al. (1982) employed HPD-PDT to treat obstructing lung tumors. Subsequently, several studies showed promising responses in early-stage cancer patients and thus PDT was recommended for patients with early-stage cancers that were inoperable, due to other complications (Dolmans et al. 2003).

The Golden Age of PDT may begin in 1993 which Photofrin® was approved for PDT of recurrent superficial papillary bladder cancer by the Canadian Health Protection Branch. This is the first official approval of PDT in the world and is regarded as a milestone in PDT history (Moan and Peng 2003). After Photofrin®, 5-aminolevulinic acid (ALA), methyl aminolevulinate, and m-THPC are also approved for treating various diseases (Moan and Peng 2003). The clinical simplicity of drug-, light-, and oxygen-based reaction has stimulated the current expansion of PDT. Since no malignancy has been found to be genetically resistant to PDT, many histologically different tumors have been treated with PDT. Clinical trials are under way to evaluate the use of PDT for cancers of the brain, breast, skin, prostate, cervix, pancreas, peritoneal cavity, and lymphatic system (Allison et al. 2006). While PDT is currently applied mostly in oncological therapy, in the future it will most likely be applied to other areas (Konopka and Goslinski 2007). As Allison et al. (2006) described PDT as the therapy that "is truly the marriage of a drug and a light", the advances of more tumor-specific photosensitizers, more convenient light delivery systems, and well-designed, randomized, and standardized controlled clinical trials may play a major role for progression of PDT in the future.

(2) Light sources

PDT involves two individually non-toxic components, light and photosensitizer,

that work together to induce cellular and tissue destruction in an oxygen-dependent manner. Human tissue transmits red light efficiently, and the longer activation wavelength of the photosensitizer results in deeper light penetration (Konopka and Goslinski 2007). Therefore, most photosensitizers are activated by red light between 630 and 700 nm, corresponding to a light penetration depth from 0.5 cm (at 630 nm) to 1.5 cm (at ~700 nm) (Salva 2002; Kubler 2005).

The light source used for PDT can be divided into two categories, the laser systems, such as argon/dye lasers, metal vapor lasers, KTP:YAG/dye lasers, and diode lasers, and non-laser light source like light emitting diode (LED) (Mang 2004). In this study, we chose the LED as the light source for our PDT. Laser machine can provide light with specific mono-wavelength; however, it is relatively complicated, bulky, heavy, expensive, and inconvenient. The LED light delivery system was simpler, smaller, lighter, cheaper, and more portable than the laser machine. Most previous PDT studies used laser light to treat a variety of premalignant and malignant human lesions; however in this study we tested the efficacy of a topical ALA-PDT using an LED red light as the light source on oral premalignant lesions.

(3) Photosensitizers

Photosensitizer is one of the key components of the PDT. An ideal photosensitizer should be non-toxic, and should display local toxicity only after activation by illumination (Konopka and Goslinski 2007). The requirements of an optimal photosensitizer include photo-physical, chemical, and biological characteristics: (i) highly selective tumor accumulation; (ii) low toxicity and fast elimination from the skin and epithelium; (iii) absorption peaks in the low-loss transmission window of biological tissues; (iv) optimum ratio of the fluorescence quantum yield (determines the photosensitizer diagnostic capabilities and plays a key role in monitoring the

photosensitizer accumulation in tissues and its elimination from them) to the interconversion quantum yield (determines the photosensitizer ability to generate singlet oxygen); (v) high quantum yield of singlet oxygen production *in vivo*; (vi) high solubility in water, injection solutions, and blood substitutes; and (vii) storage and application light stability (Konopka and Goslinski 2007). The clinically relevant guidelines for the ideal photosensitizer have been summarized by Allison et al. (2004).

Many products can serve as photosensitizers and new ones are regularly discovered. There are currently four photosensitizing agents that have received approval for PDT in various regions worldwide. They are (i) Porfimer sodium (Photofrin®), which is approved for the treatment of microinvasive endobronchial non-small cell lung cancer in patients for whom surgery and radiotherapy are not indicated; for the reduction of obstruction and palliation of symptoms in patients with completely or partially obstructing non-small cell lung cancer; for patients with total esophageal obstruction or partial esophageal obstruction where Nd:YAG laser therapy is unsuitable and also for the ablation of high-grade dysplasia in Barrett's esophagus patients who do not undergo esophagectomy; (ii) Temoporfin (Foscan®, mTHPC, bioLitec), approved in Europe for the palliative treatment of patients with advanced head and neck cancer; (iii) Vertoporphyrin (Visudyne®, QLT Vancouver BC, Canada), approved for the "wet form" of age related macular degeneration; and (iv) 5-ALA (aminolevulinic acid HCl; Levulan® Kerastick®, DUSA Inc., Valhalla, NY), approved for the treatment of actinic keratosis lesions on the face and scalp (USA) (Mang 2004).

To date, photosensitizers can be divided into three categories. The porphyrins are generally called first generation photosensitizers. Sometimes first generation labels photosensitizers developed in the 1970s and early 1980s, which by the way are the porphyrins. Photofrin® (dihematoporphyrin ether) is the most well known photosensitizer belonged to the first generation. Second generation photosensitizers,

such as ALA, benzoporphyrin derivative (BPD), mTHPC, tinethyletiopurpurin (SnET2), and talaporfin sodium (LS11) refer more to porphyrin derivatives or synthetics made from the late 1980s on. Third generation photosensitizers take available drugs and then modify with antibody conjugates, built in photo bleaching capability, biologic conjugates, etc. (Allison et al. 2004; Konopka and Goslinski 2007). However, dividing drugs into generations dose not necessarily imply that newer drugs are better than older drugs (Allison et al. 2004). Currently, only four photosensitizers are commercially available: Photofrin®, ALA, Visudyne™ (BPD; Verteporfin), and Foscan®. The first three have been approved by the FDA, while all four are in use in Europe (Konopka and Goslinski 2007). The follows are going to introduce the three most common used photosensitizers: Photofrin®, Foscan®, and 5-ALA.

Photofrin® is the most extensively studied and clinically used photosensitizer (Konopka and Goslinski 2007). It is commercially available from Axcan Pharma, Inc. and has the longest clinical history and patient track record (Allison et al. 2004). In general Photofrin® is infused at 2 mg/kg in an outpatient setting. About 48 hrs later illumination occurs generally by a diffusing fiber (which illuminates in a circumferential manner) or more rarely by a micro lens (which is unidirectional). Depending on the clinical situation, light dose of 150 J/cm^2 (lens) or 200-300 J/cm^2 (diffuser) is employed (Allison et al. 2004). The tumor is illuminated at 630 nm. At this wavelength, light penetrates 0.5 to 1.0 cm into the tissue. The drug seems reliable, easy to activate, pain-free, and non-toxic. However, it is not highly selective at 2 mg/kg. Despite of the most commonly used photosensitizer, there are several drawbacks of Photofrin®. First, it consists of about 60 compounds and therefore it is difficult to reproduce its composition. Second, its molar absorption coefficient at 630 nm is low that high concentrations of sensitizer and light must be delivered to the

tumor. Furthermore, Photofrin® itself is not very selective for tumor tissue. Finally, the major side effect of the drug is significant prolonged skin photosensitivity observed up to 6 weeks after treatment (Dolmans et al. 2003; Konopka and Goslinski 2007). The extensive normal tissue reactions can be reduced by photo-bleaching, the treatment that utilizes a lower drug dose (Biel 2002; Allison et al. 2004). Clinically, photo-bleaching allowed just enough drug to react in tumors/tissues at risk, but no PDT effect on normal tissues (Allison et al. 2004).

Foscan® [5, 10, 15, 20-meta-tetra(hydroxyphenyl)chlorin, Temoporfin, mTHPC], a potent second-generation photosensitizer, is commercially available from Biolitec Pharma Ltd. (Dublin, Ireland). In October, 2001, Foscan® was approved in the European Union, Norway, and Iceland as a local therapy for the palliative treatment of patients with advanced head and neck cancer, for whom previous therapies have failed, and who are unsuitable for radiotherapy, surgery, or systemic chemotherapy (Konopka and Goslinski 2007). Foscan® is intravenously introduced and is associated with pain. It is dosed at 0.15 mg/kg and activated with the laser light at 652 nm. Illumination usually occurs 4 days post-injection. The drug itself is highly efficient in converting light so that only 20 J/cm^2 is needed. The light sensitivity will last for a period of approximately 15 days, and appropriate light exposure precautions should be followed during this period (Allison et al. 2004; Konopka and Goslinski 2007).

There are two alternative routes of the ALA formation. One is the classic Shemin's pathway, whereby ALA results from the condensation of succinyl CoA and glycine catalyzed by ALA synthetase (Shemin and Russel 1953). This pathway takes place in the mitochondria of animals, yeasts and fungi and in some bacterial species (Fukuda et al. 2005). Another route of ALA formation, the 5-carbon route, utilizes the intact carbon skeleton of glutamate or alpha-ketoglutamate in a process requiring three enzymatic reaction and tRNA glu. These reactions take place in the stroma of

the plastid of higher plants and in greening alga.

ALA itself is not a photosensitizer but serves as the biological precursor of the photosensitizer, PpIX, in the heme biosynthesis pathway. Almost all types of cells of the human body, with the exception of mature red blood cells, are equipped with this metabolic machinery (Castano et al. 2004). The initial step in the heme synthesis pathway is the formation of ALA. This process is regulated by ALA synthetase, which is located on the matrix side of the inner mitochondrial membrane (May and Bawden 1989) as well as has the main regulatory function of the pathway (Peng et al. 1997). Subsequently, two molecules of ALA condense to form a single and also unique pyrrole ring structure called porphobilinogen (PBG). Four of these small pyrrole rings become joined to ultimately form a large ring configuration: the protoporphyrinogen IX, which is then oxidized to PpIX. PpIX is formed in the mitochondria of cells, but rapidly diffuses to other intracellular membrane sites (Castano et al. 2004). The incorporation of iron occurs under the action of ferrochelatase to form heme (Fukuda et al. 2005). There are two rate-limiting steps in this pathway. One is the first step of forming ALA from glycine and succinyl CoA, which is regulated by heme via a negative feedback mechanism. The other rate-limiting step is the conversion of PpIX to heme, and this process is controlled by ferrochelatase, which adds a ferrous iron to PpIX to form the heme. Exogenous ALA administration short-circuits the first step of porphyrin synthesis and subsequently leads to the accumulation of PpIX in the tissue. Furthermore, PpIX accumulation could be the result of a decreased conversion of PpIX to heme in tumor cells as a result of a decreased ferrochelatase activity (Kennedy et al. 1990; Kennedy et al. 1996).

There are two topical ALA-related photosensitizers that are available in the market for the treatment of cutaneous premalignant lesions. Levulan® Kerastick™

(DUSA Pharmaceuticals, Wilmington, MA, USA) is an ALA preparation that has already been approved by the Food and Drug Administration (FDA). Metvix® (PhotoCure ASA, Norway) is mainly a methyl ALA preparation that has already been approved by the European Agency for the Evaluation of Medicinal Products (EMEA).

Due to the limited depth of topical ALA, and the limited light penetration at 635 nm, the use of ALA is restricted to superficial lesions (1-2 mm). ALA is rapidly cleared from the tissues and the body within 48 hrs, and skin photosensitivity lasts less than 24 hrs (Konopka and Goslinski 2007).

(4) Mechanisms

Once the photosensitizer is applied to the tissues, it reaches its maximum concentration at various times in different tissues. Following absorption of light (photons) at specific wavelength, the photosensitizer is transformed from its ground state into a relatively long-lived electronically excited state via a short-lived excited singlet state (Henderson and Dougherty 1992). The excited triplet can undergo two kinds of reactions. Type I reaction involves electron/hydrogen transfer directly from the photosensitizer, producing ions, or electron/hydrogen removal from a substrate molecule to form free radicals. These radicals react rapidly with oxygen, resulting in the production of highly ROSs. In type II reaction, the triplet photosensitizer can transfer its energy directly to molecular oxygen (itself a triplet in the ground state), to form excited-state singlet oxygen ($^1O_2$) - a highly ROS (Dolmans et al. 2003; Castano et al. 2004; Konopka and Goslinski 2007). Both type I and type II reactions occur simultaneously, and the ratio between these processes depends on the type of sensitizer used, the concentrations of substrate and oxygen, as well as the binding affinity of the sensitizer for the substrate (Dolmans et al. 2003). ROS itself is highly active and has short half-life, only cells that are proximal to the area of the ROS

production (photosensitizer localization) are directly affected by PDT (Moan and Berg 1991). The singlet oxygen can react with electron-rich regions of many biomolecules, giving rise to oxidized species (Rosenthal and Ben-Hur 1995; Dougherty et al. 1998). It also has a very short lifetime in cells, its intracellular targets are located close to the sites where the sensitizer is located (Moan and Berg 1991). The half-life of singlet oxygen in biological systems is < 0.04 μs, and, therefore, the radius of the action of singlet oxygen is < 0.02 μm (Moan and Berg 1991).

PDT produces cytotoxic effects through photodamage to subcellular organelles and molecules. Mitochondria, lysosomes, cell membranes, and nuclei of tumor cells are considered potential targets, along with the tumor vasculature. The concentration, physicochemical properties and subcellular location of the photosensitizer, the concentration of oxygen, the appropriate wavelength and intensity of the light, and the cell-type specific properties may all influence the mode and extent of cell death. Photosensitizers that localize in mitochondria, like Photofrin®, or are produced in mitochondria, like 5-ALA-induced PpIX, are likely to induce apoptosis, while photosensitizers localized in the plasma membrane are likely to cause necrosis during light exposure (Dougherty et al. 1998).

Necrosis is a violent and quick form of degeneration affecting extensive cell populations, characterized by cytoplasm swelling, destruction of organelles and disruption of the plasma membrane, leading to the release of intracellular contents and inflammation. Apoptosis is identified in single cells usually surrounded by healthy-looking neighbors. It is characterized by cytoskeletal and organelle disruption, cell shrinkage, membrane blebbing, chromatin condensation and fragmentation, and formation of small membrane bound apoptotic bodies, which are phagocytosed by macrophages or neighboring cells.

In 1971, an Australian pathologist named John Kerr first described apoptosis. He

observed a novel form of cell death in rat liver after ligation of a branch of the portal vein. Some cells appeared to shrink and die without a swelling phase and “shrinkage necrosis” was named (Kerr 1971). Subsequently, Kerr and his colleagues further described the morphology of this form of cell death and the term “apoptosis” was introduced. The word “apoptosis” is used in Greek to describe the “dropping off” or “falling off” of petals from flowers, or leaves from trees.

There are three different pathways that can initiate apoptosis. The first is death receptor-mediated or extrinsic pathway that involves the activation of caspase-8. The second is mitochondria-mediated apoptosis or intrinsic pathway that contributes to the release of proteins by mitochondria into the cytoplasm leads to activation of caspase-9 and downstream cleavage of caspases-3, -6 or -7. The third is the endoplasmic reticulum stress pathway involves activation of caspase-12 (Almeida et al. 2004; Castano et al. 2005a).

The extrinsic pathway is triggered by stimulation of the cell surface death receptors from the tumor necrosis factor (TNF) gene family. Subsequently, the initiator caspase-8 is activated via adaptor and scaffolding proteins (Wallach et al. 1999). The intrinsic pathway, activated by cellular stress, is mediated by release of cytochrome *c* from the mitochondria leading to activation of caspase-9 (Green and Reed 1998). The Bcl-2 family is intimately involved in the regulation of the release of cytochrome *c*. The Bcl-2 family consists of two groups of proteins. The anti-apoptotic proteins such as Bcl-2 and Bcl-X_L have four Bcl-2 homology (BH) regions. The pro-apoptotic proteins comprise a multidomain group (BH1-3), such as Bax and Bak, and a BH3 only group, which includes proteins with a transmembrane domain (TM), such as Bik and Bim, or without a TM domain, such as Bid and Bad (Loro et al. 2003). Bcl-2 family members have been suggested to act through many different mechanisms. These include: (i) formation of a pore, through which cytochrome *c* and other

intermembrane proteins can escape; (ii) heterodimerization between pro- and anti-apoptotic family members; (iii) direct regulation of caspases via adaptor molecules; (iv) interaction with other mitochondrial proteins, such as voltage-dependent anion channel (VDAC) and adenosine nucleotide transporter (ANT), either to generate a pore for cytochrome *c* exit, or to modulate mitochondrial homeostasis; and (v) oligomerization to form a weakly selective ion channel (Reed 1997; Adams and Cory 1998; Gross et al. 1999; Antonsson and Martinou 2000; Hengartner 2000). Cytosolic cytochrome *c* acts as a cofactor in the formation of a complex with apoptosis protease-activating factor-1 (Apaf-1), procaspase-9, dATP/ATP termed the apoptosome that leads to the activation of caspase-9 and subsequent activation of executioner caspases and cell death commitment (Castano et al. 2005a). Apoptosis caused by stress in the endoplasmic reticulum has been shown to specifically activate caspase-12 (Nakagawa et al. 2000; Rao et al. 2001). All these pathways lead to activation of the downstream effector caspases-3, -6 and -7, which then activate or inactivate their cellular protein targets by a process of limited proteolysis (Grutter 2000). During apoptosis the cells shrink, the nuclear chromatin becomes pyknotic and condenses against the nuclear membrane, and eventually the cytoplasm and the nucleus break up into apoptotic bodies. DNA is digested at internucleosomal sites, giving rise to fragments that are multiples of 180-200 bp (Almieda et al. 2004). Cross-talk and integration between the death-receptor and mitochondrial pathways is provided by Bid (BH3-interacting domain death agonist). Caspase-8-mediated cleavage of Bid greatly increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytochrome *c* exit. Under most conditions, this cross-talk is minimal, and the two pathways operate largely independently of each other (Hengartner 2000).

There are three main mechanisms by which PDT mediates tumor destruction.

Firstly, the ROS can kill tumor cells directly. Secondly, PDT can damage the tumor-associated vasculature, leading to thrombus formation and subsequent tumor infarction. Thirdly, PDT can also activate an immune response against tumor cells (Dolmans et al. 2003).

In vivo exposure of tumors to PDT has been shown to reduce the number of clonogenic tumor cells, through direct photodamage (Henderson et al. 1985). Some parameters may limit direct tumor-cell destruction, such as non-homogenous distribution of the photosensitizer within the tumor and the availability of oxygen within the tissue that is targeted by PDT (Dolmans et al. 2003). Oxygen shortage can arise as a result of the photochemical consumption of oxygen during the photodynamic process and from the immediate effects of PDT on the tissue microvasculature (Dolmans et al. 2003). Rapid and substantial reduction in the tissue oxygen tension during and after illumination of photosensitized tissue has been reported (Tromberg et al. 1990; Pogue et al. 2001). There are two ways to overcome this problem. One is to lower the light fluence rate to reduce oxygen consumption rate (Xu et al. 2004), and the other is to fractionate the PDT light delivery to allow re-oxygenization of the tissue (Messmann et al. 1995; Pogue and Hasan 1997).

Agarwal and his colleagues first reported PDT-induced cell apoptosis in 1991 (Agarwal et al, 1991). Actually, PDT may induce a mixture of apoptotic and necrotic cell death (Oleinick et al. 2002). In general, it is believed that lower dose PDT leads to more apoptosis, while higher doses lead to proportionately more necrosis (Plaetzer et al. 2002). Previous studies have shown that the balance between apoptosis and necrosis after PDT *in vitro* depends on several parameters, including the total PDT dose (PDT dose is the product of photosensitizer concentration and light fluence), the intracellular localization of the photosensitizer, the fluence rate, the oxygen concentration, and the cell type (Castano et al. 2005a). Kessel and Luo (1998)

proposed that mitochondrial damage might be an important step in PDT-induced apoptosis. PDT-induced apoptosis leads to the rapid release of mitochondrial cytochrome *c* into the cytosol followed by activation of the apoptosome and effector caspase. Evidences indicate that a shift in the balance between the activities of pro- and anti-apoptotic members of the Bcl-2 family of proteins may decide the susceptibility of cells to PDT-mediated apoptotic death (Almeida et al. 2004). It has been shown that PDT induces selective degradation of the Bcl-2 protein, leading to apoptosis by decreasing the Bcl-2/bax ratio (Usuda et al. 2003a). With the photosensitizer localized in the plasma membrane, the photosensitization process can rapidly switch the balance towards necrotic cell death likely due to loss of plasma membrane integrity and rapid depletion of intracellular ATP (Kessel and Poretz 2000). In addition, high dose of PDT can photochemically inactivate essential enzymes and other components of the apoptotic cascade such as caspases (Castano et al. 2005a).

The significance of vascular damage was first convincingly demonstrated by Henderson et al. (1985). Microvascular collapse can be readily observed following PDT (Star et al. 1986; Henderson and Fingar 1987) and can lead to severe and persistent post-PDT tumor hypoxia (Henderson and Fingar 1987). Since the viability of tumor cells depends on the nutrient supply by the blood vessels, targeting the tumor vasculature is a promising approach to cancer treatment (Dolmans et al. 2003). The mechanisms underlying the vascular effects of PDT differ greatly with different photosensitizer (Castano et al. 2005b). They may include the platelet activation and release of thromboxane (Fingar et al. 1993a), primarily on vascular leakage (Fingar et al. 1993b), blood flow stasis via platelet aggregation (McMahon et al. 1994), or vessel constriction via inhibition of the production or release of nitric oxide by the endothelium (Gilissen et al. 1993). However, studies also showed an opposite response that the expression of potent angiogenic factors vascular endothelial growth

factor (VEGF) and cycloxygenase (COX)-2 are upregulated during PDT (Ferrario et al. 2000; Ferrario et al. 2003). These effects are presumably due to the ROS formation and hypoxia that is induced by PDT. Further studies are required to determine the long-term effects of PDT on tumor vasculature.

Canti et al. (1981) were the first who looked for and found immunological effects of PDT. Korbelik and others have carried this research forward, and it is likely that the immunological effects of PDT are significant and play a role in tumor destruction and in the prevention of tumor recurrence (Korbelik 1996). They are thought to be two aspects to the effect of PDT on the immune response against cancer: (i) anti-tumor activity of PDT-induced inflammatory cells and (ii) generation of a long-term anti-tumor immune response (Castano et al. 2005b). Differences in the nature and intensity of the inflammatory reaction between normal and cancerous tissues could contribute to the selectivity of PDT-induced tissue damage (Dolmans et al. 2003). It has been shown that the direct effects of PDT can destroy the bulk of the tumor, whereas the immune response is required to eliminate the surviving cells (Korbelik et al. 1996). The inflammatory signaling after PDT initiates a massive regulated invasion of neutrophils, mast cells, and monocytes/macrophages that may outnumber resident cancer cells (Krosl et al. 1995). Neutrophils have been shown to have a profound impact on PDT-mediated destruction of tumors, and it has been shown that depletion of neutrophils in tumor-bearing mice decreased the PDT-mediated tumor cure rate (Korbelik et al. 1996). The tumoricidal activity of monocytes/macrophages is found to be increased by PDT *in vivo* and *in vitro* (Yamamoto et al. 1992). The subsequently activated lymphocytes also contribute the tumor-specific immunity. This immune response may not only participate in the initial tumor ablation but also may be decisive in attaining long-term tumor control. In addition, the activity of tumor-sensitized lymphocytes is not limited to the original PDT-treated site but can include

disseminated and metastatic lesions of the same cancer. Thus, despite the local application of the PDT, it can have systemic effect due to the induction of an immune reaction (Castano et al. 2005b).

(5) Roles of Bcl-2 family, caspases, p53, p21, and PCNA in PDT

Bcl-2 family. The Bcl-2 family can be divided into two groups depending on their pro- or anti-apoptotic ability. At least 18 Bcl-2 proteins have been isolated (Martinou and Green 2001). Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1 possess anti-apoptotic activity, whereas Bax, Bak, Bok, Bid, Bim, Bik/Nbk, Bad, Bcl-X_S, Bmf, Hrk, Noxa and Puma are pro-apoptotic (Antonsson and Martinou 2000; Harris and Thompson 2000; Cory and Adams 2002). Studies have demonstrated that overexpression or enforced expression of Bcl-2 in different cell lines can inhibit or delay PDT-induced cell apoptosis (He et al. 1996; Chaloupka et al. 1999; Granville et al. 1999b; Vantieghem et al. 2001). Overexpression of Bcl-X_L in HL-60 cells also prevents PDT-induced cleavage of pro-caspase-3 and DNA fragmentation (Granville et al. 1998b). Overexpression of Bcl-2 and Bcl-X_L also delays morphological changes, depresses caspase activation, and limits substrate degradation in PDT-treated HeLa cells (Carthy et al. 1999). The regulatory role of Bcl-2 in PDT has also been investigated by transfection or incubation of Bcl-2 antisense sequence and that results in increased phototoxicity (Zhang et al. 1999) or sensitization (Srivastava et al. 2001) to PDT. In contrast to the above-mentioned regulatory role of Bcl-2 in PDT, studies also demonstrated that overexpression of Bcl-2 increases PDT-induced apoptotic cell death (Kim et al. 1999). This discrepancy is thought to attribute to the observed up-regulated Bax protein levels in transfected cells and to the selective degradation of Bcl-2 in photosensitized cells. A down-regulation of Bcl-2 protein levels in PDT-treated cells may have freed Bax to exert it pro-apoptotic effects, since the amount of

this protein in the cell is not changed (Kim et al. 1999). The influence of the interrelationship of the pro- and anti-apoptotic proteins in Bcl-2 family on PDT-induced cell apoptosis have been reported. Studies have indicated that a shift in the balance between the activity of pro- and anti-apoptotic members of the Bcl-2 family of proteins may decide the susceptibility of cells to PDT-mediated apoptotic death (Kessel and Castelli 2001; Srivastava et al. 2001; Xue et al. 2001; Kessel et al. 2002; Xue et al. 2003; Usuda et al. 2003b).

Bak is a multidomain pro-apoptotic member of the Bcl-2 family of proteins. In unstressed cells, Bak is located at the mitochondrial outer membrane as an inactive monomer and is complexed with other mitochondrial proteins, such as VDAC2 and Mcl-1, that function to maintain this pro-apoptotic factor in an inactive conformation (Cheng et al. 2003; Cuconati et al. 2003; Leu et al. 2004). After diverse apoptotic stimuli, Bak undergoes an activating conformational change that results in the formation of higher-order multimers; this leads to the release of cytochrome *c* and other pro-apoptogenic factors from mitochondria into the cytosol (Griffiths et al. 1999; Wei et al. 2000; Wei et al. 2001). The activity of Bak can be regulated by p53. *In vivo* and *in vitro* studies have shown that p53 can interact with Bak, but not with either Bax or Bcl-X_L (Leu et al. 2004). p53 can induce conformational change in Bak that is associated with Bak oligomerization, resulting in cytochrome *c* release from mitochondria. In addition, formation of the p53-Bak complex coincides with loss of an interaction between Bak and the anti-apoptotic Bcl-2 family member Mcl-1. Thus, p53 and Mcl-1 have opposing effects on mitochondrial apoptosis by interacting with, and modulating the activity of the death effector Bak (Leu et al. 2004). It has been shown that Bak and Bax are responsible for PDT-induced cell apoptosis (Buytaert et al. 2006a; Buytaert et al. 2006b). By immunohistochemical staining (IHC), Lai et al. (2001) demonstrated that the expression of Bak protein in nasopharyngeal carcinoma

(NPC) tends to be intensified following PDT, and those patients with favorable prognoses after PDT show a relatively strong positive reaction of Bak protein expression. They confirmed this result by the western blot analysis that PDT results in a significant increase in the expression of Bak protein in NPC. They concluded that PDT probably causes NPC cell apoptosis through an upregulation of the pro-apoptotic protein Bak expression (Lai et al. 2001). Recently, Zawacka-Pankau et al. (2007) observed that Bak levels are increased in a p53-dependent manner upon both PpIX and PDT stimulation in human HCT116 colon carcinoma cells. Therefore, Bak may play an important role in PDT-induced cell apoptosis.

Mcl-1 protein, which was first identified in 1993 (Kozopas et al. 1993), is one member of the Bcl-2 family that possesses anti-apoptotic property. It is shown to locate in mitochondria, endoplasmic reticulum, and cytosol of both MCF-7c3 (human breast cancer) and U937 (human monocyte) cells (Xue et al. 2005). In healthy cells, Mcl-1 is predominantly located in mitochondria (Yang et al. 1999; Leu et al. 2004). The anti-apoptotic members of the Bcl-2 family can protect against apoptosis by forming heterodimers with pro-apoptotic Bcl-2 family members (Yin et al. 1994; Sedlak et al. 1995; Zha et al. 1997; Bae et al. 2000). Mcl-1 contains two highly conserved regions, BH1 and BH2 domains, which have been shown to be required for the anti-apoptotic function of Bcl-2 and for heterodimerization with Bax (Yin et al. 1994). It was reported that Mcl-1 is complexed with Bak in untreated cells (Cuconati et al. 2003; Leu et al. 2004). A recent study also showed that Mcl-1 together with Bcl-X_L sequesters Bak in an inactive state in healthy cells (Willis et al. 2005). When cells undergo apoptosis, certain BH3-only proteins, such as Noxa and Bad, can displace Bak from both Mcl-1 and Bcl-X_L (Willis et al. 2005). It was also shown that p53 activation can cause disruption of the Bak-Mcl-1 complex (Leu et al. 2004). This result together with the quick degradation of Mcl-1 protein in response to many death

signals (Chao et al. 1998; Nijhawan et al. 2003; Cuconati et al. 2003) and the immediate inducibility of Mcl-1 by survival factors (Chao et al. 1998; Wang et al. 1999; Huang et al. 2000) indicate that Mcl-1 plays an important role in many cell survival and death regulatory programs (Yang-Yen 2006). Very little is known about the influence of the expression Mcl-1 protein on the efficacy of PDT. A recent study demonstrated that Mcl-1 is cleaved into a 28-kDa fragment, which has pro-apoptotic activity, in Pc 4-PDT treated lymphoid-derived cells, but not in cells of epithelial origin. The cleavage of Mcl-1 is accompanied by the activation of caspases-3, -8, and -9. This result suggests that PDT-induced rapid and extensive apoptosis in lymphoma cells may result in part from the sensitivity of their Mcl-1 to caspase cleavage, removing an important negative control on apoptosis (Xue et al. 2005).

Caspases. Caspases are known for *C*ysteine *Asp*artyl-specific Prote*ases*, which cleave their substrates at aspartic acid residues (Alnemri et al. 1996). Caspases can be thought of as the central executioners of the apoptotic pathway because they bring about most of the visible changes that characterize apoptotic cell death (Hengartner 2000). Caspases are synthesized as enzymatically inert zymogens, which are composed of three domains: an N-terminal prodomain and the p20 and p10 domains (Hengartner 2000). Three general mechanisms of caspase activation have been described. The first mechanism involves proteolytic cleavage by an upstream caspase. It is straight forward and effective, and is used mostly for activation of downstream, effector caspases like caspase-3, -6, and -7. In the second mechanism, the induced proximity is triggered by death receptors. Upon ligand binding, death receptors form membrane-bound signaling complexes. These complexes then recruit, through adapter proteins, multiple procaspase-8 molecules into close proximity that results in cross-activation. The third mechanism includes holoenzyme formation that cytochrome *c* and ATP-dependent oligomerization of Apaf-1 allows recruitment of procaspase-9 into

the apoptosome complex and then activates caspase-9 (Hengartner 2000). There are two pathways that have been described in detail in activating caspases. One of these centers is TNF family receptors, which use caspase activation as a signaling mechanism, thus connecting ligand binding at the cell surface to apoptosis induction. The other involves the participation of mitochondria, which release caspase-activating proteins into the cytosol, thereby triggering apoptosis (Reed 2000). Studies have shown that the early events occurring at the mitochondria are upstream of caspase activation in PDT-treated cells (Kessel and Luo 1999; Vantieghem et al. 2001; Lam et al. 2001). Among these, the release of cytochrome *c* from the mitochondria is the critical step for caspases activation. The release of cytochrome *c* to the cytosol in photosensitized cells induces the cleavage of the initiator caspase-9, followed by cleavage of several other caspases, including caspase-2, -3, -6, -7 and -8, in several cell types (Almeida et al. 2004). Caspase-8 is typically cleaved via the death receptor-mediated apoptotic pathway prior to cytochrome *c* release. In some cases of PDT-induced apoptosis, however, caspase-8 activation may be followed by cytochrome *c* release (Granville et al. 1998a; Granville et al. 1999a; Carthy et al. 1999). The activated caspase-8 may amplify cytochrome *c* release by cleavage of Bid into a truncated form (tBid), which then promotes cytochrome *c* release from mitochondria (Granville et al. 1998a; Granville et al. 1999a; Slee et al. 2000). In addition, the activation of caspases in photosensitized cells leads to the cleavage of a number of other cell proteins, such as Bap-31 (shuttle protein between the ER and the intermediate compartment and/or Golgi complex), DNA-dependent protein kinase, inhibitor of caspase activated DNAse (ICAD, prevents DNA fragmentation via binding to caspase-activated deoxyribonuclease), focal adhesion kinase (FAK, a kinase involved in the regulation of cell adhesion), lamins (structural components of the nuclear envelope), poly(ADP-ribose) polymerase (PARP, a DNA repair enzyme)

and Ras GTPase-activating protein (Ras-GAP, a negative regulator of the Ras signaling pathway) (Almeida et al. 2004) that may also contribute to the cell apoptosis.

p53. *p53* is a tumor suppressor gene that plays a role in cell-cycle progression, cellular differentiation, DNA repair, and apoptosis. It is the most commonly mutated gene and is altered in about 50% of all cancers, including 25-69% of oral cancers (Choi and Myers 2008). The p53 protein is present in low amounts in healthy cells, becoming up-regulated in response to various types of stress leading to cell-cycle arrest that enables DNA repair to occur (Hartwell and Kastan 1994). p53 is regarded as the "guardian of the genome" because it is essential for preventing inappropriate cell proliferation, by inducing growth arrest at different points of the cell cycle and maintaining the integrity of the genome following DNA damage or inducing apoptosis (Lane 1992). When the damage to DNA is not severe, p53 stimulates transcription of genes that mediate a delay in the G1 and G2 phases of the cell cycle to allow for DNA repair as well as reducing the expression of anti-apoptotic genes like *Bcl-2*, *Bcl-*X_L, *Mcl-1*, and *bcl-w*. If the damage is severe, apoptotic pathways will be activated and p53 will stimulate transcription of a different set of genes, including the pro-apoptotic genes *Bax*, *PIG3*, *Puma*, *Noxa*, *Fas*, and PARP (Partridge et al. 2007). The protein p53 can induce apoptosis through the intrinsic pathway by modulating the activities of many of the proteins that control the integrity of the mitochondrial pore complex as well as through the extrinsic pathway by activating the death receptors located at the cell membrane (Partridge et al. 2007). PDT-treated cells with various sensitizers up-regulate the expression of p53, but PDT-induced cell death or apoptosis does not show a significant dependence on p53 (Fisher et al. 1999; Tong et al. 2000; Hajri et al. 2002; Lee et al. 2006). Nevertheless, it was recently reported that PDT can induce cell death and apoptosis in a p53-dependent manner. Zawacka-Pankau et al. (2007) used human HCT116 colon cancer cells to test whether PpIX affects p53 activity and whether p53

plays a role in the PpIX-mediated PDT. They found that PpIX can bind to p53 tumor suppression protein *in vitro* and disrupt the interaction between p53 and its negative regulator MDM2 *in vitro* and in cells, leading to p53 accumulation and activation of its pro-apoptotic activity. Moreover, HCT116 colon cancer cells exhibit a p53-dependent sensitivity to PpIX in a dose-dependent manner. They also observed induction of p53 target pro-apoptotic genes *Puma* and *Bak* in PpIX-treated cells. In addition, p53-independent growth suppression by PpIX is detected in p53-negative cells. They concluded that PDT treatment of HCT116 cells induces p53-dependent activation of pro-apoptotic gene expression followed by growth suppression and induction of apoptosis (Zawacka-Pankau et al. 2007). Mitsunaga et al. (2007) studied ATX-S10Na(II)-mediated PDT-induced cell death using HCT116 human colon cancer cells and found that induction of early apoptosis and cell death is Bax- and p53-dependent. Furthermore, Bax and p53 are required for caspase-dependent apoptosis. The levels of anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-X_L, are decreased in Bax- and p53-independent manner. They suggested that the early apoptosis and cell death of human colon cancer cells induced by ATX-S10Na(II)-mediated PDT are mediated by p53-Bax network and low levels of Bcl-2 and Bcl-X_L proteins (Mitsunaga et al. 2007). From results of the above-mentioned studies, p53 may play an important role in the PDT-induced cell death and apoptosis.

p21. p21 is induced by p53 in response to DNA damage, and also in p53-dependent apoptosis, but not in p53-independent apoptosis (El-Deiry et al. 1994). It plays a major role in mediating the growth-suppressing and apoptosis-promoting functions of p53 (Sherr and Roberts 1995). The G1/S checkpoint of cell cycle is primarily regulated by a gene transcription complex that includes Rb and the E2F transcription factors. Rb protein is the key regulator that control G1/S cell cycle progression. It is normally hypophosphorylated and forms a complex with the

transcription factor E2F, thereby inhibiting E2F-mediated transcription of the genes that regulate DNA synthesis (Lundberg and Weinberg 1999). Phosphorylation of Rb by the cyclin-dependent kinases (cdk) 4/6 and cdk2 dissociates Rb-E2F complex, permitting transcription of genes such as c-myc, cyclin A, $p21^{WAF1}$ (Goodger et al. 1997), cyclin E, and PCNA (Partridge et al. 2007). The expression of p21 increases when a p53-mediated stress response occurs. Binding of one molecule of p21 allows cdk activity, whereas binding of two p21 molecules is inhibitory (Partridge et al. 2007). p21 can interact with cyclin/cdk, and this interaction leads to cell-cycle arrest in the G1 phase (Partridge et al. 2007; Choi and Myers 2008). p21 can also form complexes with cyclins, cdks, and PCNA. A complex formed by cyclin D/cdk2/PCNA and p21 (Xiong et al. 1993) causes a slowing down of DNA replication by inhibiting the elongation of the strand being polymerized and allowing repairs to be made to the damaged DNA (Elledge and Harper 1994; Flores-Rozas et al. 1994). p21 and PCNA are also found in complexes with cyclins A and B and their respective cdks (Li et al. 1994). Studies have shown that expression of p21 is increased in premalignant and malignant oral lesions with p53-dependent and -independent pathways, suggesting that alterations in p21 expression may be early events in oral carcinogenesis (Agarwal et al. 1998). An up-regulation in the p21 protein is observed in Pc 4-PDT-treated cells (Ahmad et al. 1998; Colussi et al. 1999). It is also noted a down-regulation of five members of the E2F transcription factor family, and of their heterodimeric partners DP1 and DP2 in Pc 4-PDT-treated cells (Ahmad et al. 1999). These results indicate that, in PDT-treated cells, up-regulation of p21 can inhibit cdk activities and in turn reduce phosphorylation of Rb protein as well as the activity of E2F transcription factor, leading to a cell arrest and a subsequent apoptotic cell death.

PCNA. PCNA, a co-factor of DNA polymerase δ, is a 36 kDa nuclear protein and plays an important role in DNA synthesis, DNA repair, cell cycle progression, and cell

proliferation (McCormick and Hall 1992). It is generally detected in cell nuclei between the G1 and M phases of the cell cycle (Celis and Celis 1985; Hall et al. 1990) and is a useful immunohistochemical marker of cell proliferation because its expression and distribution correlate with cellular proliferation rates and DNA synthesis (Jaskulski et al. 1988; Zuber et al. 1989). In vertebrate cells, PCNA is crucially regulated by the tumor suppressor protein p21 (Dotto 2000). Binding of p21 to PCNA inhibits replication *in vitro* and *in vivo* by blocking activity of PCNA to stimulate polymerases (Waga et al. 1994; Rousseau et al. 1999). PCNA is also crucial for the balance of survival and cell death. In response to UV irradiation, PCNA has a tenfold binding ability to p33(ING1b), an isoform of the ING1 tumor suppressor, inducing cell apoptosis. PCNA also displays an anti-apoptotic activity through interaction with proteins of the Gadd45 family, which is implicated in growth control, apoptosis, and DNA repair (Moldovan et al. 2007). Recent data reveal that p53 and its negative regulator MDM2 can interact with PCNA (Banks et al. 2006). Depletion of PCNA results in an accumulation of p53, suggesting that PCNA might indeed contribute directly to p53 stability (Moldovan et al. 2007). Expression of PCNA has been investigated in oral cancers and oral premalignant lesions. The PCNA labeling indices (LIs) vary from 11.6% to 65.6% in oral cancers, from 6.1% to 28.9% in oral leukoplakia, and from 17.4% to 44.9% in oral epithelial dysplasia (Chiang et al. 2000). PCNA expression is usually used to determine the treatment efficacy after PDT at different intervals (Uehara et al. 1999; Uehara et al. 2001; Togashi et al. 2006) or light intensities (Romanco et al. 2005). However, the influence of PCNA expression in tissue samples before PDT on the clinical outcome of PDT has not yet been reported.

(6) ALA-PDT for head and neck lesions

Grant et al. (1993) first used PDT with orally administered ALA to treat 4 patients

with advanced OSCC. They found tumor necrosis in 3 of 4 patients treated with PDT. Fan et al. (1996) used PDT with systemic ALA to treat 6 oral cancer patients. They observed a reduction in the size of the cancer in 5 of 6 patients after PDT, but only 2 showed a complete regression of the cancer. For oral precancerous lesions, Fan et al. (1996) treated 12 oral dysplastic lesions with systemic ALA-PDT. All 12 dysplastic lesions showed a significant regression to normal or less dysplastic after PDT. Kubler et al. (1998) treated 12 OL lesions with PDT after local application of 20% ALA cream. Of the 12 OL lesions, complete response (CR) was found in 5, partial response (PR) in 4, and no response (NR) in 3. Sieron et al. (2001; 2003) treated 17 OL lesions with PDT after topical application of 10% ALA ointment or emulsion in two separate studies. A CR was observed in 14 out of 17 OL lesions. The results of above-mentioned investigations indicate that PDT with either systemic or topical ALA may be an effective treatment modality for oral precancerous lesions and at least has some beneficial effects on OSCCs.

The PDT treatments for oral precancerous and cancerous lesions mentioned above were all conducted by laser light sources. In this study, we tested the efficacy of topical ALA-PDT on oral precancerous lesions using LED red light as the light source.

C: Cryotherapy

(1) History

The word “cryotherapy” is often used interchangeably with “cryosurgery”, which uses freezing temperatures to achieve specific effects on tissues (Kuflik 1994). The Egyptians were the first to use cold temperatures for trauma and inflammation (Ishida and Ramos-e-Silva 1998). Cryotherapy was used by Hippocrates to stop hemorrhage and pain (Dawber et al. 1992). In the nineteenth century, Baron Lorrey observed its utility in anesthesia and sedation for amputation in soldiers (Shepherd and Dawber

1982; Dawber et al. 1992). In 1851, Dr. James Arnott of Brighton, England, was the first to report on the therapeutic use of low temperatures in malignant diseases by means of a salt/ice mixture applied to breast neoplasm (Leopard 1975). In 1877, Cailletet of France and Pictet of Switzerland obtained the liquefaction of oxygen and carbon monoxide (Ishida and Ramos-e-Silva 1998). In 1892, James Dewar of Great Britain designed the first vacuum flask to facilitate storage and handling of cryogens (Ishida and Ramos-e-Silva 1998). Campbell White, a New York City physician, is considered to be the first cryosurgeon. In 1899, he reported clinical trials using liquid air as a spray or with a cotton swab to treat some benign lesion and early epitheliomas (White AC 1899; White AC 1901). In 1907, Whitehouse of New York described the use of a spray bottle, although he found this technique difficult and stopped using it in favor of a cotton swab (Whitehouse 1907). Dr. William Pusey of Chicago used carbon dioxide (-78.5℃) to treat various skin lesions (Pusey 1907). Liquid oxygen (-182.9℃) came into clinical use in the 1920s. However, it is hazardous because it is combustible (Irvine and Turnacliff 1929). Following World War II, liquid nitrogen (-196℃) became commercially available (Freiman and Bouganim 2005). In 1950, Allington introduced liquid nitrogen into clinical practice using cotton swabs dipped in liquid nitrogen to treat various non-neoplastic skin lesions (Allington 1950), but the destructive depth of this technique was limited to a few millimeters. Modern cryosurgery began through the collaborative work of Cooper and Lee in 1961. They designed an apparatus using liquid nitrogen in a closed system that permitted continuous and rapid extraction of heat from tissues. They described the use of a liquid nitrogen probe to produce temperatures of approximately -190℃ (Cooper and Lee 1961). Between 1961 and 1970, other cryosurgical apparatuses were developed using liquid nitrogen and other cryogenic agents, including nitrous oxide, carbon dioxide, argon, ethyl chloride, and fluorinated hydrocarbons (Torre 1975). Zacarian

and Adham used solid copper cylinder disks that were cooled by immersion in liquid nitrogen before application to the skin and accomplished greater depth of destruction than cotton-tipped applicators (Zacarian and Adham 1966). In 1965, Douglas Torre, a dermatologist, developed a nitrogen spray device that could also be used with cryoprobe tips of various shapes and sizes (Torre 1968). In 1967, Setrag Zacarian introduced a hand-held self-pressurized device (Zacarian 1969). With advances of the cryosurgical apparatus, deeper destruction of tissue could be achieved. Thus malignant and benign lesions became amenable to cryosurgical management. Cryotherapy now is a well-established treatment modality for a wide variety of benign and malignant skin lesions, with novel uses continually described (Freiman and Bouganim 2005).

(2) Mechanisms

There are several distinct mechanisms by which tissues may be damaged by freezing (Leopard 1975). They can be divided into direct and indirect effects.

Direct Effects

1. Cellular disruption (Ice crystal formation): Ice crystal formation occurs in both intracellular and extracellular fluids where the rate of freezing is rapid (greater than 5℃ per second) or particularly at the probe tip where the cooling rate approaches minus 70℃ per second (Whittaker 1972). Cellular disruption is dependent upon both the cooling rate and the final temperature (Reade 1979). The more rapid the cooling rate, the larger the ice crystals formed. Large ice crystals produce physical disruption of cell membranes (Holden and Saunders 1973).

2. Cellular dehydration and electrolyte disturbance: Within the outer zones of the iceball where the temperature is about -10℃, the cooling rate approximates to -1/2℃ per second (Leopard 1975). At such a rate, freezing occurs in the extracellular space

only because the cell membrane offers a barrier to prevent intracellular ice formation. Thus the intracellular water passes out of the cells and become trapped in the ice. These effects result in dehydration and physical shrinkage of the cells. In addition, the concentrations of electrolytes both inside and outside the cell membranes increase to levels that become toxic to cellular function (Leopard 1975). Both of these changes lead to cell damage that may be irreversible (Reade 1979). It is interesting to know why the cell interior remains in the liquid phase at the same temperature as the frozen extracellular space. It is thought that there is a lower rate of crystal nucleation and growth inside the cells due to differences in composition maintained by cell membrane function. Moreover, there are numerically fewer centers for crystallization within cells. In addition, because the intracellular water passes out of the cell membrane, there is little residual free water for intracellular ice formation (Leopard 1975).

3. Enzyme inhibition. The functions of cell enzyme are at a narrow temperature. Sudden cooling of the cells acts as an inhibitor that leads to modification of cellular metabolism. This may increase a cell's vulnerability to irreversible damage (Leopard 1975; Reade 1979).

4. Protein changes. Lipoprotein complexes in both the cell membranes and mitochondria are denatured and this subsequent interferes with semipermeability and cell function (Holden and Saunders 1973).

5. Thawing effects. During the rewarming of the frozen tissue, intracellular ice crystallization occurs with resultant cell damage (Leopard 1975). Furthermore, because of the increased intracellular electrolyte concentration within the frozen cells and the increased permeability of the cell membranes during thawing, extracellular water will transfer into cells with consequent swelling and possible rupture (Leopard 1975; Reade 1979). The longer the period of thawing, the greater the period of time

over which these damaging effects can occur (Reade 1979).

Indirect Effects

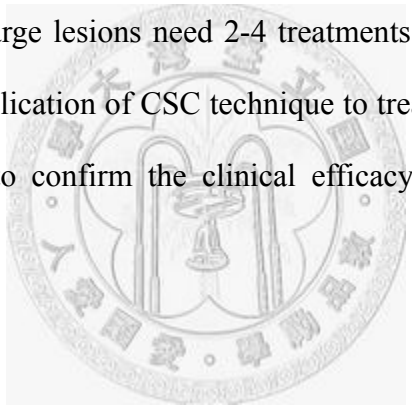
1. Vascular effects. It was demonstrated that local vascular change is unlikely to cause primary cell damage (Whittaker 1972). It occurs within a few hours of freezing that blood flow through small vessels is reduced and becomes more severe of all but the wide vessels by 24 hours. The vascular changes may include spacing of endothelial cells, clumping and adherence of platelets that lead to infarction, and a delayed increase in vascular permeability (Leopard 1975; Reade 1979). Therefore, it is thought that the vascular changes are responsible for the delayed indirect damaging effects of freezing by stasis of blood flow and microthrombus formation, finally leading to ischemic necrosis (Gill et al. 1970).

2. Immunological effects. The immunological response after cryosurgery might add to the destruction of a lesion. The effects might due to the antigenic substances within the normal cells are released when cells are killed by freezing or because of the alterations in molecular structure during freezing that render normal cell components antigenic (Leopard 1975).

(3) Cotton-swab cryotherapy technique

Previous studies have reported the clinical outcomes of treating OL lesions with cryotherapy (Emmings et al. 1967; Miller 1969; Sako et al. 1972; Chapin and Burkes 1973; Bekke and Baart 1979; Gongloff et al. 1980; Gongloff and Gage 1983; Al-Drouby 1983; Yeh 2000). Sako et al. (1972) treated 60 patients with OL lesions by a special cryosurgical unit and all OL lesions showed CR after 1-5 treatments. Chapin and Burkes (1973) used cryotherapy with a gold cryoprobe to treat 4 patients with dysplastic and non-dysplastic OL lesions and observed CR of all lesions after 1-2 treatments. Bekke and Baart (1979) used cryotherapy with a probe to treat 35 OL

lesions from 24 patients; all lesions showed CR after 1-4 treatments. All the above-mentioned cryotherapy techniques used closed systems that need a complex and delicate apparatus. In 1993, Toida et al. first described cotton-swab cryotherapy (CSC) technique for treating 18 patients with oral mucous cysts (Toida et al. 1993). This technique was performed by direct application of liquid nitrogen to the lesion with a cotton swab. Although CSC is a simple, easy method that does not require sophisticated skill and equipments (Toida et al. 1993), there are few studies using CSC technique for treating oral lesions (Toida et al. 1993; Yeh 1998; Yeh 2000a; Yeh 2000b; Yeh 2003). Yeh (2000a) used CSC technique to treat 102 oral lesions including 25 OL lesions. He found that the small and superficial oral lesions show CR after one treatment, and the deep, large lesions need 2-4 treatments to achieve a CR. Because the data on the clinical application of CSC technique to treat OL lesions were limited, it required more studies to confirm the clinical efficacy of this technique on OL lesions.



V. SPECIFIC GOALS

1. To test the efficacy of the new ALA-PDT protocol on OVH, OL, and OEL lesions.
2. To assess whether the OL lesions treated with topical ALA-PDT twice a week had a significantly better clinical outcome than OL lesions treated once a week by the same treatment protocol.
3. To assess whether OEL lesions treated with topical ALA-PDT once a week had a significantly better clinical outcome than OL lesions treated once a week by the same PDT protocol.
4. To assess what clinicopathological parameters of OVH lesions could influence ALA-PDT treatment outcomes.
5. To assess whether expression of certain apoptosis-related proteins in OVH, OL lesions may influence the clinical outcome of topical ALA-PDT.
6. To test the clinical efficacy of CSC on OL lesions and assess what clinicopathological parameters of OL lesions could influence CSC treatment outcomes.

VI. MATERIALS AND METHODS

A: Photodynamic therapy

Patients

Thirty-six patients (35 men and one women, aged 32-79 years, mean age 51 ± 11 years) with OVH, 97 patients (90 men and 7 women, aged 26-77 years, mean age 51 ± 12 years) with OL, and 16 male patients (aged 36-68 years, mean age 51 ± 9 years) with a total of 20 OEL lesions were recruited from the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital (NTUH) from November 2001 to July 2007. Clinical diagnosis of OVH was made when patients showed characteristic single or multiple elevated verrucous lesions without marginal induration on the oral mucosa. OL is defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other diseases. OEL is defined as an intermixed red and white lesion that cannot be characterized clinically or pathologically as any other diseases. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens taken from the characteristic part of the OVH, OL or OEL lesion at the patient's first visit. The histological criteria for a diagnosis of OVH were: (i) epithelial hyperplasia with parakeratosis or hyperkeratosis and verrucous surface, and (ii) no invasion of the hyperplastic epithelium into the lamina propria as compared to adjacent normal mucosal epithelium. OL or OEL lesions were diagnosed as epithelial hyperplasia with either hyperkeratosis or parakeratosis, when no dysplastic cell was found in the hyperplastic epithelium. Furthermore, OL or OEL lesions were diagnosed as mild, moderate or severe dysplasia, when enough dysplastic cells were present in the basal one third, in the basal two thirds, or in more than the basal two thirds but not the complete layer of the oral epithelium, respectively. Mild, moderate or severe dysplasia detected in OVH lesions was also recorded.

Clinicopathological data of 36 OVH, 97 OL, and 16 OEL patients are shown in Table 1. Of the 36 OVH lesions, 24 were located at the buccal mucosa, 8 at the labial mucosa, 3 at the soft palate, and 1 at the alveolar mucosa. The most common site for 97 OL lesions was the buccal mucosa (62 cases), followed by the tongue (21 cases), alveolar mucosa (6 cases), gingiva (4 cases), labial mucosa (2 cases), and soft palate (2 cases). Of 20 OEL lesions, 18 were located at the buccal mucosa, 1 at the lower labial mucosa and 1 at the alveolar mucosa. Of the 16 patients with OEL lesions, 13 had a solitary lesion, one had three separate OEL lesions at bilateral buccal mucosa and lower labial mucosa, respectively; one had two separate OEL lesions at the bilateral buccal mucosa, respectively; and one had two separate OEL lesions at the right buccal mucosa and lower alveolar mucosa, respectively. All patients received biopsy before treatment except one OVH patient, because the lesion was too small. All the 35 OVH lesions fitted the histological criteria of OVH described above, but 2 also showed mild dysplasia, 4 moderate dysplasia, and 3 severe dysplasia. The most common pathological diagnosis for 97 OL lesions was epithelial hyperplasia with hyperkeratosis (45 cases), followed by epithelial hyperplasia with parakeratosis (30 cases), mild desplasia (13 cases), and moderate dysplasia (9 cases). Of the 20 OEL lesions, 4 demonstrated mild dysplasia, 12 moderate dysplasia, 3 severe dysplasia, and 1 carcinoma *in situ*.

The color of the 36 OVH lesions were either pink (n = 22) or white (n = 14). The greatest diameter of the OVH lesion varied from 0.5 to 3.1 cm with a mean diameter of 1.7 ± 0.7 cm. The clinical apperance was of a mass in 17 lesions, of a plaque in 16 lesions, or of a combination type with peripheral plaque and central mass in 3 lesions. The surface keratin thickness of each OVH lesion was measured from hematoxylin and eosin (H&E)-stained tissue section by a built-in microscopic meter and expressed as a mean of 5 measurements from 5 randomly-selected areas. The surface keratin

thickness of 35 OVH lesions varied from 20 to 200 μm with a mean thickness of 50 ± 38 μm.

All the male patients were both AQ chewers and smokers, but the 7 female patients had none of both habits. All male patients stopped chewing AQs, but most of them continued to smoke cigarettes with a reduced number during the treatment period. Informed consent was obtained from each patient before biopsy procedure and before topical ALA-PDT. This study was reviewed and approved by the Human Investigation Review Committee at the NTUH.

ALA preparation

The gel used for preparation of 20% ALA contained 25% Pluronic F127 and 1% Carbopol 971P. Pluronic F127 was obtained from BASF (Mount Olive, NJ, USA). Carbopol 971P was a gift from BF Goodrich. Double concentrated gels of Pluronic F127 and Carbopol 971P in distilled water were separately prepared prior to mixing. Prior to use, 200 mg of ALA was mixed with 800 mg of the gel and packed into a needleless 1-cc syringe and used within 3 hrs after the preparation.

Fluorescence spectroscopy

ALA diffusing into lesional epithelial cells is metabolized into PpIX in the mitochondrial matrix and cytosol. To monitor the conversion of ALA into PpIX and to determine when the PpIX reached its peak level in the lesional epithelial cells, ALA-induced PpIX fluorescence spectra at 410-nm excitation were measured 1, 1.5, 2, and 2.5 hrs after local application of 20% ALA onto each oral lesion. This fluorescence spectroscopy was performed by using a handheld optical fiber probe attached to a spectrofluorometer (SkinSkan, JC Inc., Urbana, IL, USA) at the patient's second visit. A monochromator with a 150 W ozone-free Xenon lamp provided the excitation light.

The excitation light was guided to illuminate samples by one arm of a Y-type quartz fiber bundle, and the emission fluorescence was collected by another arm of the fiber bundle. The emission fluorescence spectra were collected from 450 nm to 750 nm in 3-nm increments during the measurement. A personal computer was used to analyze the fluorescence spectral data of each oral lesion in order to find out the time point at which the PpIX reached its peak level in the lesional epithelial cells. This time point was the most appropriate occasion for each particular oral lesion to deliver the light treatment. The kinetics study was done once for each patient and 68 of the 149 patients participated in the kinetics study. After analyzing the fluorescence spectral data of each oral lesion, we found that the PpIX reached its maximum level in the lesional epithelial cells approximately 1.5-2 hrs after local ALA application. In addition, there was no significant difference in the kinetics of PpIX among OL, OEL and OVH lesions. In general, the PpIX reached its maximum level in the epithelial cells of more keratotic oral lesions approximately 2 hrs after local ALA application and in the epithelial cells of less keratotic oral lesions approximately 1.5 hrs after local ALA application. Therefore, the subsequent light treatments were set at 1.5-2 hrs after topical application of ALA onto all lesions.

PDT

Topical ALA-PDT was performed starting from the patient's third appointment. At the day of treatment, 20% ALA was applied to each oral lesion upon patient's arrival. To avoid the dilution of the ALA by the saliva, three cotton rolls were placed onto the orifices of bilateral Stensen's and Wharton's ducts before ALA application. In addition, the patient was asked to lie on the dental chair with the mouth being opened and the doctor performing the treatment controlled the leaked saliva by intermittent suctions during the initial 10 min after topical application of ALA. The LED light

source for ALA-PDT consists of a high power LED with the wavelength centered at 635 ± 5 nm and a bandwidth of 20 nm. This LED light source was fed by a small DC-power supply. Via optical lens, the LED red light was coupled to an optical fiber with a diameter of 10 mm. The irradiation intensity of LED device at the lesion surface was 100 mW/cm^2 with a spot size of 1 cm^2 at a distance of 0.5 cm. The material cost of this LED light device is less than US $700 (Tsai et al. 2004). The treatment protocol was composed of five 3-minute and one 100-second irradiations with an LED red light at 635 ± 5 nm separated by five 3-minute rests for a total of 1000 seconds (fluence rate, 100 mW/cm^2; light exposure dose, 100 J/cm^2) 1.5-2 hrs after topical application of 20% ALA onto oral lesions. If possible, the whole lesion was irradiated using one spot of light. If the lesion was too large to be covered by one spot of light or separated into multiple portions, several spots of light were applied until the whole lesion was completely illuminated. The doctors performing the treatment held the LED light device during the whole treatment period. The tip of the LED light device was kept as close to the surface of oral lesion as possible. Thirty-six OVH, 65 OL, and 20 OEL lesions were treated once a week and 32 OL lesions were treated twice a week by this topical ALA-PDT protocol. When the lesion was treated once a week, it was treated on every Friday morning. When the lesion was treated twice a week, it was treated on every Wednesday and every Friday morning. In 26 patients (12 OVH, 10 OL, and 4 OEL patients) with severe throbbing pain or marked burning sensation during PDT, light treatments were carried out under local anesthesia using 2% lidocaine with the patients in clear consciousness. In 36 patients (14 OVH, 16 OL and 6 OEL patients) with severe post-PDT pain, analgesics (acetaminophen, 500 mg/tablet, one tablet three or four times a day) were prescribed to the patients after PDT. The post-PDT pain often occurred 1 or 2 hrs after PDT and lasted for 24-48 hrs. Those patients with pain during treatment usually had concomitant post-PDT pain. As

stated by the patients, our prescription could well control the post-PDT pain. Repeated PDT was performed once or twice a week for at most 8 treatments.

Clinical evaluation

Lesion response was characterized as follows: CR, lack of detectable lesion confirmed by clinical evaluation; PR, reduction of lesion by at least 20% in diameter; and NR, reduction of lesion by less than 20% in diameter. All lesion responses were evaluated at the completion of initial 8 treatments of ALA-PDT. If the lesion showed CR after less than 8 treatments of PDT, the PDT was ended and the patient was arranged for a follow-up schedule once a month. If the lesion showed PR or NR even after 8 treatments of PDT, the patients might choose a plan of total excision of the residual lesion, a follow-up schedule once a month, or a continuous topical ALA-PDT once or twice a week. Clinical photographs were taken at each patient's visit to evaluate the clinical outcome of PDT.

Statistical analysis

The difference in efficacy of PDT treatment between two different groups was assessed for statistical significance by chi-square test (2 rows by 3 columns). The difference in the mean treatment number of topical ALA-PDT to achieve a CR or in the mean surface keratin thickness of OVH lesions between any two different groups was assessed for statistical significance by Student's *t*-test. Generalized linear regression model and multivariate analysis were performed to assess which clinicopathological parameter was the most important or independent factor that could influence the PDT treatment outcome. A *P* value of less than 0.05 is considered statistically significant.

Immunohistochemical staining

Patients and specimens

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 58 patients (55 men and 3 women, mean age 47 years, range 24-76 years) including 18 patients (17 men and 1 women, mean age 47 years, range 28-76 years) with OVH and 40 patients (38 men and 2 women, mean age 46 years, range 24-71 years) with OL. Clinicopathological data of the 58 patients are shown in Table 2. The most common site for 18 OVH and 40 OL lesions was the buccal mucosa (39 cases), followed by the tongue (7 cases), labial mucosa (5 cases), gingiva (3 cases), soft palate (3 cases), and floor of mouth (1 case). Histological diagnosis of OVH or OL lesions was based on microscopic examination of H&E-stained tissue sections. Three OVH lesions also had mild OED and one moderate OED. Moreover, 10 OL lesions also had mild OED, one moderate OED, and 3 severe OED. The surface keratin thickness of each lesion was measured from H&E-stained tissue sections by a built-in microscopic meter and expressed as a mean of five measurements from five randomly-selected areas. The mean surface keratin thickness of 18 OVH and 40 OL lesions was 33 ± 29 μm and 61 ± 44 μm, respectively. All patients received topical ALA-PDT for their OVH and OL lesions at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital (NTUH), Taipei, Taiwan during the period from 2003 to 2006. Specimens were obtained from incisional biopsy of the characteristic part of the lesion before PDT. All the 18 OVH showed CR. Of the 40 OL lesions, 12 showed CR, 13 PR, and 15 NR.

All the male patients were both AQ chewers and smokers, but the three female patients had none of both habits. All male patients stopped chewing AQs, but most of them continued to smoke cigarettes with a reduced number during the treatment period. Informed consent was obtained from each patient before biopsy procedure and

before PDT.

Immunohistochemical staining

All the specimens for IHC were fixed in 10% neutral formalin, embedded in paraffin, and cut in serial sections of 4 μm. IHC 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 0.1 M citrate solution (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 10 min. After washing in 10 mM Tris-buffered saline (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 the primary antibodies. The specificity, source, dilution and positive controls of the primary antibodies used for IHC are listed in Table 3. After washing in TBS, sections were treated with either biotinylated goat anti-rabbit IgG (for anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-Mcl-1, and anti-Bak) or biotinylated goat anti-mouse IgG (for anti-human p53, anti-$p21^{WAF1}$, and anti-PCNA) 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. TBS or normal rabbit or mouse serum instead of primary antibodies was used for negative controls.

A brown cytoplasmic staining was counted as positive for caspase-3, caspase-8, caspase-9, Mcl-1, and Bak proteins, whereas a brown nuclear staining was considered as positive for Mcl-1, p53, p21, and PCNA proteins. The sections were initially

scanned at low power, at least three high-power fields were then chosen randomly, and at least 1,000 tumor cells were counted for each case. The labeling indices (LIs) of cytoplasmic or nuclear staining of each antibody were counted as a ratio of immunostaining-positive cells to the total number of cells counted. The staining intensity (SI) of cells was graded as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong staining. The labeling score (LS) was defined as LI × SI. An eyepiece graticule was used to ensure that all tumor cells were evaluated once only. 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 90%. The slides with discrepant assessments were reevaluated, and a consensus was reached in all cases.

B: Cryotherapy

Patients

Forty-seven patients (44 men and 3 women; mean age, 53 ± 13 years; range, 26-87 years) with a total of 60 OL lesions were recruited from the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital (NTUH) from July 2005 to July 2007. OL is defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other diseases. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens taken from the characteristic part of the OL lesion at the patient's first visit. OL lesions were diagnosed as epithelial hyperplasia with either hyperkeratosis or parakeratosis, when no dysplastic cell was found in the hyperplastic epithelium. Furthermore, OL lesions were diagnosed as mild, moderate or severe dysplasia, when enough dysplastic cells

were present in the basal one third, in the basal two thirds, or in more than the basal two thirds but not the complete layer of the oral epithelium, respectively. The surface keratin thickness of each OL lesion was measured from H&E-stained tissue sections by a built-in microscopic meter and expressed as a mean of 5 measurements from 5 randomly-selected areas.

All the 44 male patients were both AQ chewers and smokers, but the 3 female patients had none of both habits. All male patients stopped chewing AQs, but most of them continued to smoke cigarettes with a reduced number during the treatment period. Informed consent was obtained from each patient before biopsy procedure and before cryotherapy.

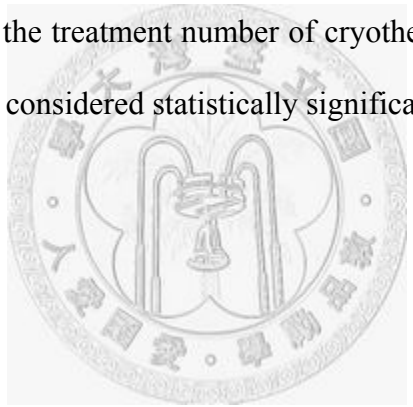
Cotton-swab cryotherapy

We used a CSC technique to treat OL lesions. Two kinds of cotton swab with the diameter of 4 mm and 7 mm were used for cryotherapy depending on the size of the lesion. In brief, the lesion site was air dried before treatment to prevent the cotton swab sticking to the lesional oral mucosa. The cotton swab was dipped into liquid nitrogen for at least 5 seconds and applied to the lesion with pressure for 20 seconds to form an ice ball and then allowed thawing for another 20 seconds. Four consecutive freezing-thawing cycles were performed for the same area of the lesion. High-power suction was used for control of saliva and vapor fog as well as for an increase of visibility. When the OL lesion was larger than 49 mm^2, each area of 49 mm^2 was treated separately until the entire lesion was completely treated. Most patients (n = 45) could tolerate the pain induced by the treatment. In two patients with severe pain, cryotherapy was carried out after topical application of 2% xylocaine jelly. All the 60 OL lesions were treated once 2 weeks until a CR of the lesion. Choline salicylate gel was prescribed to each patient after cryotherapy to control the post-cryotherapy pain if

necessary. In 17 patients with moderate to severe post-cryotherapy pain, analgesics (acetaminophen, 500 mg/tablet, one tablet three or four times a day) were prescribed to the patients after treatment. Clinical photographs were taken at each patient's visit to evaluate the clinical outcome of cryotherapy.

Statistical analysis

The relationship between the treatment number of cryotherapy to achieve a CR and each of clinicopathological parameters was assessed for statistical significance by chi-square test (2 rows by 3 columns). Multivariate analyses by Poisson regression model were used to assess which clinicopathological parameter was an independent factor that could influence the treatment number of cryotherapy to achieve a CR. A *P* value of less than 0.05 was considered statistically significant.



VIII. RESULTS

A: Photodynamic therapy

Oral verrucous hyperplasia

All the 36 OVH lesions were treated with topical ALA-PDT once a week. At the day of treatment, the lesions were topically applied with 0.1-0.6 (average, 0.25) cc of 20% ALA solution, depending on the size of the lesions. All the 36 OVH lesions showed CR (Table 4 and Figures 1A-D) after an average of 3.8 (range, 1-6) treatments of ALA-PDT. In addition, no recurrence of the 36 OVH lesions was found after a follow-up period of 17-67 (mean, 37) months.

Correlation between the mean treatment number of topical ALA-PDT to achieve a CR and the clinicopathological parameters of 36 OVH lesions is shown in Table 5. We found that the mean treatment numbers of PDT to achieve a CR for OVH lesions with an clinical apperance of a mass (2.9 ± 1.3), with the greatest diameter < 1.5 cm (3.0 ± 1.3), with the pink color (3.2 ± 1.4), with epithelial dysplasia (3.1 ± 1.5), or with the surface keratin layer ≦ 40 μm (3.4 ± 1.4) was significantly less than for OVH lesions with an clinical apperance of a plaque or a combination type (4.7 ± 1.1, $P = 0.000$), with the greatest diameter ≧ 1.5 cm (4.3 ± 1.4, $P = 0.011$), with the white color (4.8 ± 1.1, $P = 0.000$), without epithelial dysplasia (4.2 ± 1.3, $P = 0.043$), or with the surface keratin layer > 40 μm (4.8 ± 0.7, $P = 0.003$), respectively (Table 5). However, the mean treatment number for OVH lesions was not correlated with the age of the patient and the lesion location (Table 5). By generalized linear regression model, we further discovered that the clinical apperance of the lesion was the most important factor influencing the PDT treatment number, followed by the size, color, the presence or absence of epithelial dysplasia, and the surface keratin thickness of the lesion. Multivariate analysis showed that only the clinical apperance of OVH lesions was an independent factor ($P = 0.0069$) that could influence the treatment number of

PDT to achieve a CR.

Histologic examination demonstrated that the mean surface keratin thickness of OVH lesions was significantly thinner in lesions with pink color (31 ± 10 μm) or with epithelial dysplasia (28 ± 4 μm) than in lesions with white color (79 ± 46 μm, $P = 0.000$) or without epithelial dysplasia (58 ± 41 μm, $P = 0.037$), respectively.

Oral leukoplakia

The 65 OL lesions treated with topical ALA-PDT once a week showed CR in 5, PR in 33, and NR in 27 (Table 4). The former 5 OL lesions required 0.1-0.2 (mean, 0.12) cc of 20% ALA solution and an average of 3.8 (range, 1-7) treatments of ALA-PDT to achieve CR of the lesions (Figures 1E-G). One of the 5 OL lesions with CR recurred 6 months after the first PDT. This lesion was treated with topical ALA-PDT twice a week and regressed completely after 4 treatments. The lesion recurred again 11 months later. It was treated with topical ALA-PDT twice a week again but the lesion failed to response after 8 treatments. Therefore, this lesion was finally excised by surgery. The remaining 4 OL lesions with CR showed no recurrence after a follow-up period of 3-36 (mean, 24.3) months. Of the 33 PR patients, 8 received further treatment with topical ALA-PDT once a week, 2 received further treatment with topical ALA-PDT twice a week, 4 chose total excision of the lesion, and the remaining 19 chose a follow-up schedule once a month. No significant improvement of the lesion was found in 8 patients who received further treatment with topical ALA-PDT once a week for at least 8 treatments, but the two lesions treated with topical ALA-PDT twice a week showed a significant reduction in size after 8 treatments. Of the 27 NR patients, 6 received further treatment with topical ALA-PDT once a week, 5 received further treatment with topical ALA-PDT twice a week, 10 chose total excision of the lesion, and the remaining 6 chose a follow-up schedule

once a month. The 6 NR patients received further treatment with topical ALA-PDT once a week showed no significant improvement of the lesion after at least 8 treatments. However, the 5 NR patients received further treatment with topical ALA-PDT twice a week demonstrated a shift from NR to PR after 8 treatments. Of the 25 follow-up OL lesions including 19 PR and 6 NR lesions, 18 enlarged slightly after a follow-up period of 3-34 (mean, 22.2) months, and 7 showed no significant change in size.

The 32 OL lesions treated with topical ALA-PDT twice a week showed CR in 11 and PR in 21 (Table 4). The former 11 OL lesions required 0.1-0.3 (mean, 0.22) cc of 20% ALA solution and an average of 4 (range, 2-6) treatments of ALA-PDT to achieve CR of the lesions. Two of the 11 OL lesions recurred 9 and 11 months after complete regression of the lesion, respectively. The remaining 9 OL lesions with CR showed no recurrence after a follow-up period of 8-20 (mean, 13.1) months. The latter 21 OL lesions showed PR even after 8 treatments of ALA-PDT with 0.2-0.3 (mean, 0.21) cc of 20% ALA solution. Compared to the 65 OL lesions treated once a week by the same treatment protocol of topical ALA-PDT, the 32 OL lesions treated twice a week had a significantly better clinical outcome than the 65 OL lesions treated once a week ($P < 0.001$, Table 4). Of the 21 PR patients, 10 received further treatment with topical ALA-PDT twice a week and the remaining 11 chose a follow-up schedule once a month. Of the 10 OL lesions receiving further treatment, 9 revealed a significant reduction in size after 4-10 (mean, 7.1) months of treatment, and 1 showed complete regression after 4 months of treatment. Of the 11 follow-up OL lesions, 10 enlarged slightly after a follow-up period of 9-24 (mean, 16.5) months, and 1 showed no significant change in size.

We further analyzed the correlation between the lesion location or pathological diagnosis and clinical outcomes of 97 OL lesions treated by ALA-PDT. We found that

22 OL lesions with dysplasia were prone to have good clinical outcomes compared to 75 OL lesions without dysplasia after topical ALA-PDT treatment (marginal significance, $P = 0.052$). However, there was no significant difference in the clinical outcome between the 21 OL lesions at the tongue and 76 OL lesions at other oral mucosal sites after topical ALA-PDT treatment ($P > 0.05$) (Table 6).

Oral erythroleukoplakia

The 20 OEL lesions treated with topical ALA-PDT once a week showed CR in 17 and PR in 3 (Table 4). The former 17 OEL lesions required 0.1-0.3 (mean, 0.20) cc of 20% ALA solution and an average of 3.8 (range, 2-8) treatments of ALA-PDT to achieve CR of the lesions. No recurrence of 17 OEL lesions with CR was found after a follow-up period of 2-62 (mean, 18) months. Compared to the 65 OL lesions treated once a week by the same treatment protocol of topical ALA-PDT, the 20 OEL lesions treated once a week had a significantly better clinical outcome than the 65 OL lesions treated once a week ($P = 0.000$, Table 4).

Immunohistochemical staining

Representative microphotographs of IHC for different apoptosis-associated proteins are shown in Figure 2. In all lesions, Bak protein showed a condense immunostaining in the cytoplasm closely opposite to the cell nucleus (Figure 2A). Bak protein expresssion was evenly distributed throughout the epithelium of OVH lesions with a median SI of 3, but was scattered in the epithelium of OL lesions with a median SI of 2.

Both cytoplasmic and nuclear Mcl-1 stains were found in OVH and OL lesions. Cytoplasmic Mcl-1 immunostaining was found prominently in the lower one-third of the epithelium, whereas the nuclear Mcl-1 immunostaining was noted majorly in the

upper two-thirds of the epithelium in OVH lesions. In OL lesions, nuclear Mcl-1 staining was scattered throughout the epithelium, whereas cytoplasmic Mcl-1 staining was found in the lower two-thirds of the epithelium (Figure 2B). The median Mcl-1 SI was 2 and 3 for OVH and OL lesions, respectively.

Cytoplasmic caspase-3 immunostaining was evenly distributed throughout the epithelium of OVH and OL lesions (Figure 2C). The median caspase-3 SI was 1 in both lesions. Positive caspase-8 immunostaining was found in the cytoplasm of nearly all epithelial cells in both OVH and OL lesions (Figure 2D). The median caspase-8 SI was 2 in OVH lesions and 1 in OL lesions. Caspase-9 was mainly expressed in the cytoplasm of the lower two-third epithelial cells in both OVH and OL lesions (Figure 2E). The median caspase-9 SI was 2 in OVH lesions and 1 in OL lesions.

Expression of p53 was demonstrated in the nuclei of basal and parabasal epithelial cells in both OVH and OL lesions (Figure 2F), with a grade 2 p53 SI in the CR group and a grade 1 p53 SI in the PR or NR group. Nuclear p21 immunostaining was evenly scattered throughout the epithelium in both OVH and OL lesions (Figure 2G). The median p21 SI was 2 in the CR group and 1 in the PR or NR group. Nuclear PCNA immunostaining was noted mainly in lower one-third epithelial cells in both OVH and OL lesions (Figure 2H). The median PCNA SI of was 3 in both OVH and OL lesions.

Comparisons of the means of surface keratin thickness, Bak/Mcl-1 LS ratio and LS between CR group and PR or NR group in 18 OVH and 40 OL lesions are summarized in Table 7. The mean surface keratin thickness was significantly less in CR group (41 ± 32 μm) than in PR or NR group (64 ± 47 μm, $P = 0.036$). There was a significant difference in the mean Bak/Mcl-1 LS ratio between CR (2.1 ± 1.8) and PR or NR (0.7 ± 0.7) groups ($P = 0.020$) and in the mean Bak LS between CR (210 ± 117%) and PR or NR (115 ± 108%) groups ($P = 0.003$). However, no significant

difference in the Mcl-1, caspase-3, caspase-8, caspase-9, p53, p21 or PCNA LS was demonstrated between the CR and PR or NR groups (*Ps* > 0.05).

B: Cryotherapy

A total of 60 OL lesions from 47 patients were treated by the CSC technique. Thirty-five patients had 1 OL lesion, eleven had 2, and one had 3 OL lesions on their oral mucosae. The buccal mucosa (n = 33) was the most common site for OL lesions, followed by the tongue (n = 11), palate (n = 6), alveolar mucosa (n = 4), gingiva (n = 4), lip (n = 1), and floor of mouth (n = 1). The surface area of OL lesions varied from 0.1 to 6.5 cm^2 with a mean of 1.8 ± 1.5 cm^2. Histopathological examination revealed that 19 OL lesions had epithelial hyperplasia with parakeratosis, 13 epithelial hyperplasia with hyperkeratosis, 26 mild dysplasia, and 2 moderate dysplasia. The surface keratin thickness of 60 OL lesions varied from 25 to 240 μm with a mean thickness of 55 ± 32 μm. The mean surface keratin thickness was significantly thicker in OL lesions without epithelial dysplasia (63 ± 39 μm, $P = 0.029$) or on the tongue (78 ± 63 μm, $P = 0.005$) than in OL lesions with epithelial dysplasia (45 ± 18 μm) or on other oral mucosal sites (49 ± 16 μm).

Hyperemia and edema of the treated area occurred immediately after cryotherapy. Local swelling and bullous formation became evident during the following 2-3 days. Subsequently, superficial necrosis occurred and the lesion was covered by a thin layer of yellowish pseudomembrane. Epithelization of each treated area was complete after 10-14 days, depending on the location and area of the lesion. The healing process of all lesions was uneventful and most lesions healed within 2 weeks without any complications.

All the 60 OL lesions showed CR without scar formation after an average of 6.3 (range, 1-17) treatments of cryotherapy (Figure 3). The correlation between the

treatment number of CSC to achieve a CR and each of the clinicopathological parameters of 60 OL lesions is shown in Table 1. We found that the treatment number of CSC to achieve a CR for OL lesions on the oral mucosal sites other than the tongue, < 2 cm^2, with epithelial dysplasia, or with the surface keratin thickness < 55 μm was significantly less than that for OL lesions on the tongue ($P = 0.003$), $\geqq 2$ cm^2 ($P = 0.024$), without epithelial dysplasia ($P = 0.033$), or with the surface keratin thickness $\geqq 55$ μm ($P = 0.045$), respectively (Table 8). Multivariate analyses by Poisson regression model showed that only the location ($P = 0.000176$) and area ($P = 0.021280$) of the OL lesions were independent factors that could influence the treatment number of cryotherapy to achieve a CR (Table 9).

The follow-up period for 60 OL lesions varied from 2 to 27 (mean, 14) months. During the follow-up period, 5 OL lesions (3 on the buccal mucosa, 1 on the tongue, and 1 on the gingiva) recurred 1-5 (mean, 2.1) months after cryotherapy. All the 5 recurred OL lesions achieved a CR again after an average of 3.4 (range, 2-5) treatments of cryotherapy.

VIII. DISCUSSION

Photodynamic therapy

PDT with systemically or topically administered ALA has been used for the treatment of dysplastic Barrett's esophagus (Barr et al. 1996; Gossner et al. 1998; Ackroyd et al. 2000), cervical intraepithelial neoplasia II (Bodner et al. 2003), carcinoma *in situ* of the urinary bladder (Waidelich et al. 2001), Bowen's disease of the skin (Gold and Goldman 2004), and oral premalignant lesions (Fan et al. 1996; Kubler et al. 1998; Sieron et al. 2001; Sieron et al. 2003) with promising clinical outcomes. Fan et al. (1996) treated 12 oral dysplastic lesions with systemic ALA-PDT. All 12 patients with dysplasia showed regression of the lesion to normal or less dysplastic. Kubler et al. (1998) treated 12 OL lesions with PDT after local application of 20% ALA cream every 30 minutes for 2 hrs. They observed CR in 5 OL lesions, PR in 4 OL lesions, and NR in 3 OL lesions 3 months later. Sieron et al. (2001; 2003) treated 17 OL lesions with PDT after topical application of 10% ALA ointment or emulsion in two separate studies. A CR was observed in 14 out of 17 OL lesions.

This study showed that the topical ALA-PDT we used was very effective for OVH lesions and all the 36 OVH lesions could be completely ablated after less than 7 treatments once a week. We suggest that the successful clinical outcome of OVH lesions treated by this ALA-PDT may be due to a new ALA preparation, a new topical ALA-PDT protocol used, and the characteristic features of the OVH lesion itself. The 20% ALA (w/w) preparation used in our studies was a liquid form at room temperature; it became a gel form at body temperature upon contacting the lesional oral mucosa due to a thermoresponsive sol-gel transition of the vehicle. The gel form of ALA preparation was adhesive to the oral mucosa and partially resistant to the dilution of the saliva. This characteristic feature of our ALA preparation, in turn, helped the absorption of ALA from the mucosal surface. Furthermore, the verrucous

appearance of the OVH lesion provided a large area for good retention of ALA on the surface and the less keratotic epithelium of the OVL lesion than the OL lesion also provided a more permeable surface layer for good absorption of ALA into cells. In addition, we divided the 1000-second light treatment course into 5 periods of 180 seconds and a period of 100 seconds in our studies. These 6 periods of light treatment were interrupted by 5 periods of 3-minute rest. Because an efficient PDT needs sufficient and continuous supply of new PpIX and oxygen, multiple 3-minute stops were supposed to give the opportunities for tissues to regenerate new PpIX and to obtain new oxygen. This, in turn, resulted in a successful clinical outcome for OVH lesions with this new protocol of topical ALA-PDT.

It is easy to understand that smaller OVH lesions need less mean PDT treatment number to achieve a CR than larger OVH lesions, and more ALA can diffuse into the OVH lesions with thinner surface keratin layer than those with thicker surface keratin layer. This study showed that OVH lesions with pink color or dysplasia had thinner surface keratin layer than those with white color or no dysplasia, respectively. Therefore, pink OVH lesion may retain more ALA than white OVH lesions, and OVH lesions with dysplasia may absorb more ALA than those without dysplasia. In addition, dysplastic OVH lesions usually have more permeable epithelium (due to wide intercellular spaces of the dysplastic epithelium) than non-dysplastic OVH lesions. Therefore, ALA may be easier to diffuse into dysplastic than non-dysplastic epithelia. Furthermore, the dysplastic epithelium may retain more ALA than the hyperplastic epithelium, and the thinner keratin layer may only have a minimal effect on the reduction of the light intensity. The sufficient photosensitizers and light dose finally resulted in a better clinical outcome for OVH lesions with pink color, epithelial dysplasia, and thinner surface keratin layer than for those with white color, no epithelial dysplasia, and thicker surface keratin layer, respectively.

Mass-typed OVH lesions are highly protruded lesions and are usually situated on a smaller base area than the plaque-typed or combination-typed OVH lesions. In our experience, the purely mass-typed lesions or the central masses of combination-typed lesions usually showed complete regression after one or two treatments of PDT. In contrast, the purely plaque-typed lesions or the residual peripheral plaques of combination-typed lesions might need more PDT treatments to achieve a CR. Because PDT can damage the tumor-associated vasculature and mass-typed OVH lesions with a smaller base area usually have limited blood supply, these mass-typed OVH lesions are easily eradicated by PDT probably through a combined mechanism of ROS-mediated tumor cell killing and endothelial cell destruction followed by thrombus formation and tumor infarction (Dolmans et al. 2003).

This study found that topical ALA-PDT once a week was not an effective treatment modality for OL lesions. The 65 OL lesions treated with ALA-PDT once a week demonstrated CR in 5 (8%), PR in 33 (51%), and NR in 27 (41%). Moreover, 8 PR and 6 NR OL lesions showed no significant reduction in size after at least 8 further treatments with topical ALA-PDT once a week. The unsatisfactory clinical outcome for OL lesions after topical ALA-PDT once a week could be due to the outer form and inner structure of OL lesions. Compared to OVH lesions, OL lesions have relatively smooth surface and are covered by thicker keratin layer. Some ALA gel on the relatively smooth surface of OL lesions may be easy to dilute out by the saliva. The thicker keratin layer on the surface of OL lesions may interfere with the diffusion and absorption of ALA into epithelial cells. Furthermore, the thicker keratin layer may reduce the light intensity and prevent sufficient light from reaching the underlying lesional epithelial cells. The insufficient photosensitizers and light dose finally resulted in an unsatisfactory clinical outcome for our OL lesions.

This study showed that the 32 OL lesions treated with topical ALA-PDT twice a

week had a significantly better clinical outcome than 65 OL lesions treated once a week by the same treatment protocol. Furthermore, 2 PR and 5 NR OL lesions in the topical ALA-PDT once-a-week group and 9 PR OL lesions in the topical ALA-PDT twice-a-week group exhibited a significant reduction in size after at least 8 further treatments with topical ALA-PDT twice a week. One PR OL lesion in the topical ALA-PDT twice-a-week group even showed complete regression after 4 months of further treatment with topical ALA-PDT twice a week. These findings demonstrate that twice-a-week treatment modality is better than the once-a-week treatment modality for OL lesions. Previous studies and our clinical observation revealed that ALA-PDT-treated oral lesions became ulcerated 2-3 days after PDT and re-epithelization of the ulcerated area happened 5-6 days after PDT (Grant et al. 1993a; Fan et al. 1996). We suggest that the second PDT performed 5 days after the first PDT may destroy the adjacent residual lesional epithelial cells, prevent the regrowth of the OL lesion, and finally result in a significant reduction of the lesional size.

In the present study, we found that only one third of the 32 OL lesions could achieve a CR after treatment with topical ALA-PDT twice a week. Although 94% (16/17) of PR or NR OL lesions receiving further treatment with topical ALA-PDT twice a week demonstrated a significant reduction in the lesional size, they were difficult to achieve a CR even after 10 months of treatment. This suggests that those OL lesions showing PR or NR after 8 treatments of ALA-PDT are probably resistant to further treatment with the ALA-PDT protocol used in this study. Furthermore, approximately 78% (28/36) follow-up OL lesions showed a slight increase in the size of the residual lesion during the follow-up period. These findings indicate that it is necessary to develop a new treatment modality for those OL lesions that fail to response to topical ALA-PDT either once or twice a week.

The clinical outcomes of ALA-PDT-treated OL lesions in our studies were not as

good as those in the studies of Kubler et al. (1998) and Sieron et al. (2001; 2003). We suggest that the discrepancies in the treatment results may be due to differences in the ALA preparation (different bases and concentrations), the number of ALA application (single or multiple), the incubation period (1.5-5 hrs), the light source (laser or LED), the light delivery protocol (continuous or fractionated), and the number of treatment (single or multiple).

In this study, we found that 22 OL lesions with mild or moderate dysplasia had a better clinical outcome than 75 OL lesions without dysplasia after topical ALA-PDT treatment (marginal significance, $P = 0.052$). In addition, all 20 OEL lesions had epithelial dysplasia (4 with mild, 12 with moderate, and 3 with severe dysplasia, and 1 with carcinoma *in situ*) and their clinical outcomes after topical ALA-PDT treatment were better than 65 OL lesions treated by the same PDT protocol. We suggest that the better clinical outcome for dysplastic OL or OEL lesions after topical ALA-PDT may be due to the inner structure of these dysplastic oral lesions. Compared to non-dysplastic OL lesions, dysplastic oral lesions have less keratotic epithelial surface as well as thinner and more permeable epithelium (due to wide intercellular spaces of the dysplastic epithelium). Therefore, ALA may be easy to diffuse into these dysplastic epithelia resulting in a good absorption of ALA into dysplastic epithelial cells. Furthermore, the dysplastic epithelium may retain more ALA than the hyperplastic epithelium and the thinner keratin layer may only have a minimal effect on the reduction of the light intensity. The sufficient photosensitizers and light dose finally resulted in a better clinical outcome for dysplastic OL or OEL lesions than for non-dysplastic OL lesions. We suggest that all dysplastic oral lesions can be treated first by topical ALA-PDT because it can be repeatedly used without causing any side effects, it has no cutaneous photosensitivity even exposure of patients' skin immediately after treatment, and it results in little scar formation and a good clinical outcome.

Photofrin® (porfimer sodium) and Foscan® (meta-tetrahydroxyphenylchlorin, mTHPC or temoporfin) are two more potent photosensitizers than ALA, because these two agents have greater penetration depth of 5 mm and 10 mm, respectively, than that (2 mm) of ALA. Systemic Photofrin®-mediated PDT can be used to eliminate premalignant lesions in 9, 10 dimethyl 1, 2 benzanthracene (DMBA)-treated hamsters (Kingsbury et al. 1997) and to eradicate OL lesions in patients with "field cancerization" of the oral cavity (Grant et al. 1993b). Furthermore, systemic Foscan®-mediated PDT is effective for treatment of vulval intraepithelial neoplasia type III (Campbell et al. 2004). Since Photofrin® and Foscan® have the characteristic feature of greater penetration depth, they are usually used to treat oral cancers rather than oral precancerous lesions. In addition, no topical forms of these two drugs are available in the market. Therefore, it is difficult to compare the clinical efficacy of PDT using these two photosensitizers with that of topical ALA-PDT.

This study showed that the surface keratin thickness could significantly affect the treatment outcome of topical ALA-PDT for OVH and OL lesions. This result is consistent with that the mean treatment numbers of PDT to achieve a CR for OVH lesions with the surface keratin layer $\leqq$ 40 μm was significantly less than for OVH lesions with the surface keratin layer $>$ 40 μm as previously discussed.

IHC study discovered a significantly higher expression of Bak protein in OVH and OL lesions of the CR group than in those of the PR or NR group. Bak is a multidomain pro-apoptotic protein of the Bcl-2 family. Upon apoptotic stimuli, Bak can promote cell apoptosis via forming multimers that lead to the release of cytochrome *c* and other pro-apoptogenic factors from mitochondria into the cytosol (Griffiths et al. 1999; Wei et al. 2000; 2001). Therefore, the higher expression of Bak in OVH and OL lesions of the CR group may lead more cells susceptible to apoptotic insults. Lai and co-workers studied the Bak protein expression in biopsy specimens of

nasopharyngeal carcinoma (NPC) before and after PDT by IHC and found that 75% of the patients had a significant upgrade expression of Bak protein in their tumor tissues after PDT. NPC patients with a high Bak protein expression in post-PDT tumor tissues showed favorable prognoses after PDT (Lai et al. 2001). This result indicates that PDT probably causes NPC cell apoptosis through an upregulation of the Bak protein expression. Our study showed a significantly higher Bak protein expression in the CR group than in PR or NR group. The result indicates that Bak protein may play a crucial role in the topical ALA-PDT-induced cell apoptosis and may also a potential biomarker that is able to predict the treatment outcome of ALA-PDT for OVH and OL lesions.

It was reported that Mcl-1 is complexed with Bak in untreated cells (Cuconati et al. 2003; Leu et al. 2004) and can sequesters Bak in an inactive state together with Bcl-X_L in healthy cells (Willis et al. 2005). In addition, evidences have indicated that a shift in the balance between the activities of pro- and anti-apoptotic members of the Bcl-2 family of proteins may decide the susceptibility of cells to PDT-mediated apoptotic death (Almeida et al. 2004). In this study, there was no significant difference in Mcl-1 protein expression between CR and PR or NR groups. However, OVH or OL lesions with a high Bak/Mcl-1 ratio showed a better clinical outcome after topical ALA-PDT. The result indicates that the ratio of the pro-apoptotic to anti-apoptotic protein is also an important factor that can determine the clinical outcome of PDT.

Caspases is thought to be the central executioners in the apoptotic pathway. The extrinsic apoptotic pathway can trigger caspase-8, whereas caspase-9 can be activated through the intrinsic pathway. These two caspases, also called initiator caspase, can then activate the downstream caspase-3, the effector caspase, to trigger cell apoptosis. Nevertheless, this study showed no significant differences in the LS of caspase-8, -9, or -3 between the CR and PR or NR groups. These results indicate that although

caspases-8, -9, and -3 are central executioners in the apoptotic pathway, their expression levels in the oral lesional epithelium before PDT only have a minimal influence on the treatment outcome of topical ALA-PDT for OVH and OL lesions.

This study observed that there was no significant difference in the p53 LS between CR and PR or NR groups. Although cells treated by PDT with various sensitizers up-regulate the expression of p53, PDT-induced cell death or apoptosis does not show a significant dependence on p53 protein expression (Fisher et al. 1999; Tong et al. 2000; Hajri et al. 2002; Lee et al. 2006). Nevertheless, Zawacka-Pankau and collegues (2007) demonstrated that PpIX-mediated PDT-induced cell death and apoptosis functions in a p53-dependent manner. They found that PpIX-mediated PDT-treated HCT116 colon cancer cells can induce p53-dependent activation of pro-apoptotic gene expression followed by growth suppression and induction of apoptosis (Zawacka-Pankau et al. 2007). Another study also showed that an early apoptosis and cell death of HCT116 cells induced by ATX-S10Na(II)-mediated PDT are regulated by p53-Bax network and low levels of Bcl-2 and Bcl-X_L proteins (Mitsunaga et al. 2007). Base on the above-mentioned findings, p53 protein may play an assistant role in contribution to the ALA-PDT-induced cell death and apoptosis via up- and down-regulation of different Bcl-2 family proteins.

This study showed a higher p21 LS in lesions of the CR group than in lesions of PR or NR group. However, no statistically significant difference was found between the two groups. p21 is induced by the wild-type p53 in response to DNA damage (El-Deiry et al. 1994). It also plays a major role in mediating the growth-suppressing and apoptosis-promoting functions of p53 (Sherr and Roberts, 1995). Thus, p21, like p53, may possibly act as an assistant role in the topical ALA-PDT-induced cell death and apoptosis.

PCNA is crucially regulated by the tumor suppressor protein p21 in vertebrate

cells (Dotto 2000). Binding of p21 to PCNA inhibits replication *in vitro* and *in vivo* by blocking activity of PCNA to stimulate polymerases (Waga et al. 1994; Rousseau et al. 1999). The present study displayed the PCNA LS did not significantly affect ALA-PDT outcomes on OVH and OL lesions. We suggest that PCNA may only has a minimal effect on the topical ALA-PDT-induced cell apoptosis.

Cryotherapy

Previous studies have reported the clinical outcomes of treating OL lesions with cryotherapy (Emmings et al. 1967; Miller 1969; Sako et al. 1972; Chapin and Burkes 1973; Bekke and Baart 1979; Gongloff et al. 1980; Gongloff and Gage 1983; Al-Drouby 1983; Yeh 2000). Sako et al. (1972) treated 60 patients with OL lesions by a special cryosurgical unit and all OL lesions showed CR after 1-5 treatments. Chapin and Burkes (1973) used cryotherapy with a gold cryoprobe to treat 4 patients with dysplastic and non-dysplastic OL lesions and observed CR of all lesions after 1-2 treatments. Bekke and Baart (1979) used cryotherapy with a probe to treat 35 OL lesions from 24 patients; all lesions showed CR after 1-4 treatments. Yeh (2000a) used CSC technique to treat 102 oral lesions including 25 OL lesions. He demonstrated that the small and superficial lesions showed CR after one treatment, and the deep, large lesions needed 2-4 treatments to achieve a CR. In this study, we found that OL lesions could be eradicated by a mean of 5.4 treatments of CSC for those < 2.0 cm^2 and by a mean of 8.1 treatments of CSC for those $\geqq 2.0$ cm^2. The more treatment number needed to achieve a CR in this study compared to those reported in previous studies might be due to the fact that we used a more conservative cryotherapy technique in which a constant low temperature was difficult to maintain in an open system. Moreover, we used a high-power suction to control the vapor fog; in this situation the liquid nitrogen may easily evaporate from the cotton swab during cryotherapy.

Therefore, the destruction of the lesional tissue treated by our CSC technique might be milder than those treated by special cryotherapy equipments that could maintain a lower and more constant temperature in lesional tissues during the whole treatment procedure.

Most animal tissues freeze at -2.2℃ and the cell death occurs at the temperature of -20℃ (Fraser and Gill 1967). The mechanisms for cell destruction after cryotherapy are complex with a combination of direct and indirect effects being involved (Reade 1979). The direct effects consist of ice crystals formed in both extracellular and intracellular fluid, cellular dehydration, toxic intracellular electrolyte concentration, inhibition of enzymes, protein damage, thawing effects that cause cell to vacuolate, swell and rupture (Leopard 1975, Reade 1979), and thermal shock injury to cells (Reade 1979). The indirect effects include vascular changes that lead to ischemic necrosis of the treated tissue and immunological responses that cause cell damage through cytotoxic immune mechanism (Leopard 1975, Reade 1979).

Our study showed that OL lesions on the tongue needed more treatment number than those on other oral mucosal sites. The dorsal surface of tongue is a specialized mucosa covered predominantly by a thick keratinized stratified squamous epithelium. This study demonstrated that OL lesions on the tongue had a significantly thicker surface keratin layer than those on other oral mucosal sites. Furthermore, the tongue is a highly vascular organ which is rich in capillary plexus in the long connective tissue papillae that are closer to the mucosal surface (Nanci 2003). We suggest that the thicker surface keratin layer on the tongue lesions than on other oral mucosal lesions may act as a more effective barrier against the transmission of low temperature into the underlying lesional epithelial cells. In addition, the rich blood supply in the lamina propria of the tongue may also contribute to the warming effect against tissue freezing and promote the rate of tissue recovery including the regrowth of the lesion after

cryotherapy.

This study found that small OL lesions needed significantly less treatment number of cryotherapy than large OL lesions. Pogrel et al. (1996) showed that the center of an iceball produced by liquid nitrogen on soft tissues ultimately undergo necrosis, while the margin of the iceball usually does not obtain enough low temperature to induce effective tissue necrosis. In this study, a large OL lesion was divided into several small areas that were treated separately. Compared to treatment of a large lesion as a whole by special cryotherapy equipments, treatment of a large lesion part by part by our CSC technique left multiple insufficiently treated margins and finally resulted in the need of more treatment number of cryotherapy to achieve a CR.

This study demonstrated a significantly less treatment number of cryotherapy to achieve a CR for OL lesions with dysplasia than for OL lesions without dysplasia. Histological examination revealed a thinner surface keratin layer on dysplastic OL lesion than on non-dysplastic OL lesions. Thus, the need of less treatment number to achieve a CR for dysplastic OL lesions may partly due to the thinner surface keratin layer on top of dysplastic OL lesions. Indeed, the present study also showed that OL lesions with surface keratin thickness < 55 μm needed significantly less treatment number of cryotherapy to achieve a CR than those with surface keratin thickness $\geqq$ 55 μm. Moreover, the dysplastic oral epithelium usually had wider intercellular spaces and contained more proliferating cells than non-dysplastic oral epithelium (Chiang et al. 2000). Wider intercellular space resulted in more extracellular ice crystals formed during cryotherapy and thus enhanced the tissue destruction effect caused by cryotherapy. In addition, proliferating epithelial cells are more sensitive to cryotherapy-induced tissue damage than resting epithelial cells. These two reasons could also explain why dysplastic OL lesions needed less treatment number of

cryotherapy to achieve a CR than non-dysplastic OL lesions.

We conclude that for OVH lesions less than or equal to 3.1 cm in greatest diameter, complete regression of the lesions can be achieved by less than 7 treatments of topical ALA-PDT once a week. Furthermore, the PDT treatment outcome for OVH depends on the clinical apperance, size, color, epithelial dysplasia, and surface keratin thickness of the lesion. The clinical apperance of OVH lesions is the only independent factor affecting the PDT treatment outcome. OL lesions treated twice a week have a significantly better clinical outcome than OL lesions treated once a week. In addition, OEL lesions treated once a week have a significantly better clinical outcome than OL lesions treated once a week. The LS of Bak and the Bak/Mcl-1 ratio in tissue sections of OVH and OL lesions before PDT can be used as biomarkers to predict the PDT treatment outcomes. For OL lesions with the surface area ranging from 0.1 to 6.5 (mean, 1.8) cm^2, CR of the lesion can be achieved by less than 7 CSC treatments in average. OL lesions on oral mucosal sites other than the tongue, with dysplasia, and with thinner surface keratin layer needed significantly less treatment number of CSC to achieve a CR than OL lesions on the tongue, without dysplasia, and with thicker surface keratin layer, respectively. Therefore, CSC technique may be a simple, safe, easy, and conservative treatment modality for OL lesions that fail to respond to other treatment modalities.

IX. CONCLUSIONS

1. Topical ALA-PDT is a very effective treatment modality for OVH lesions.
2. For OVH lesions less than or equal to 3.1 cm in greatest diameter, complete regression of the lesions can be achieved by less than 7 treatments of topical ALA-PDT once a week.
3. The PDT treatment outcome for OVH lesions depends on the clinical apperance, size, color, epithelial dysplasia, and surface keratin thickness of the lesion. The clinical apperance of OVH lesions is the only independent factor affecting the PDT treatment outcome.
4. OL lesions treated with topical ALA-PDT twice a week have a significantly better clinical outcome than OL lesions treated with the same PDT protocol once a week.
5. OEL lesions treated with topical ALA-PDT once a week have a significantly better clinical outcome than OL lesions treated with the same PDT protocol once a week.
6. The LS of Bak and the Bak/Mcl-1 ratio in tissue sections of OVH and OL lesions before PDT can be used as biomarkers to predict the PDT treatment outcomes.
7. CSC technique is a simple, safe, easy, and conservative treatment modality for OL lesions that fail to respond to other treatment modalities.
8. For OL lesions with the surface area ranging from 0.1 to 6.5 (mean, 1.8) cm^2, CR of the lesion can be achieved by less than 7 CSC treatments in average.
9. OL lesions on oral mucosal sites other than the tongue, with dysplasia, and with thinner surface keratin layer needed significantly less treatment number of CSC to achieve a CR than OL lesions on the tongue, without dysplasia, and with thicker surface keratin layer, respectively.

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XII. TABLES

Table 1. Clinicopathological data of 36 patients with oral verrucous hyperplasia (OVH), 97 patients with oral leukoplakia, and 16 patients with a total of 20 oral erythroleukoplakia lesions

	Oral verrucous hyperplasia (n = 36)	Oral leukoplakia (n = 97)	Oral erythrolekoplakia (n = 20)	Total (n = 153)
Age				
20-29	0	1	0	1
30-39	7	16	2	25
40-49	9	32	8	49
50-59	14	24	6	44
60-69	4	12	4	20
70-79	2	12	0	14
Gender				
Men	35	90	20	145
Women	1	7	0	8
Location				
Buccal mucosa	24	62	18	99
Tongue	0	21	0	21
Labial mucosa	8	2	1	11
Alveolar mucosa	1	6	1	8
Gingiva	0	4	0	4
Soft palate	3	2	0	5
Pathological diagnosis				
EH with hyperkeratosis	0	45	0	45
EH with parakeratosis	0	30	0	30
Mild dysplasia	0	13	4	17
Moderate dysplasia	0	9	12	21
Severe dysplasia	0	0	3	3
Carcinoma in situ	0	0	1	1
OVH[a]	26	0	0	26
OVH with mild dysplasia	6	0	0	6
OVH with moderate dysplasia	2	0	0	2
OVH with severe dysplasia	1	0	0	1

EH = epithelial hyperplasia.

[a]Biopsy was not done in one OVH patient, because the lesion was too small.

Table 2. Clinicopathological data of 18 oral verrucous hyperplasia (OVH) and 40 oral leukoplakia (OL) lesions which were examined by immunohistochemistry

	OVH (n = 18)	OL (n = 40)	Total (n = 58)
Age			
20-29	1	2	3
30-39	5	10	15
40-49	3	13	16
50-59	6	10	16
60-69	2	3	5
70-79	1	2	3
Gender			
Men	17	38	55
Women	1	2	3
Location			
Buccal mucosa	11	28	39
Tongue	0	7	7
Labial mucosa	3	2	5
Gingiva	2	1	3
Soft palate	2	1	3
Floor of mouth	0	1	1
Pathological diagnosis			
OVH	14	0	18
OVH with mild dysplasia	3	0	3
OVH with moderate dysplasia	1	0	1
OVH with severe dysplasia	0	0	0
EH with hyperkeratosis	0	23	23
EH with parakeratosis	0	3	3
Mild dysplasia	0	10	10
Moderate dysplasia	0	1	1
Severe dysplasia	0	3	3

EH = epithelial hyperplasia.

Table 3. The specificity, source, dilution, and positive controls of the primary antibodies used for immunohistochemistry

Antibody	Source	Dilution	Positive control
Polyclonal rabbit anti-caspase-3	Lab Vision	1: 100	Tonsil
Polyclonal rabbit anti-caspase-8	Lab Vision	1: 100	Tonsil
Polyclonal rabbit anti-caspase-9	Lab Vision	1: 50	Tonsil
Epitope specific rabbit anti-Mcl-1	Lab Vision	1: 100	Tonsil
Epitope specific rabbit anti-Bak	Lab Vision	1: 100	Tonsil
Monoclonal mouse anti-human p53	Dako	1: 25	Oral squamous cell carcinoma
Monoclonal mouse anti-p21^{WAF1}	Lab Vision	1: 100	Colon carcinoma
Monoclonal mouse anti-PCNA	Lab Vision	1: 400	Tonsil

Table 4. Clinical outcomes of 36 oral verrucous hyperplasia (OVH) lesions, 65 oral leukoplakia (OL) lesions, and 20 oral erythroleukoplakia (OEL) lesions treated by the topical ALA-PDT once a week and of 32 OL lesions treated by the topical ALA-PDT twice a week

Group of lesion	Clinical outcome		
	Complete response	Partial response	No response
36 OVH lesions treated once a week	36	0	0
65 OL lesions treated once a week	5	33	27
32 OL lesions treated twice a week	11	21	0
20 OEL lesions treated once a week	17	3	0

Chi-square test showed a significant difference in clinical outcome between the 65 OL lesions treated once a week and 32 OL lesions treated twice a week ($P < 0.001$) as well as between the 65 OL lesions treated once a week and 20 OEL lesions treated once a week ($P = 0.000$).

Table 5. Correlation between the mean treatment number of topical ALA-PDT to achieve a complete response and the clinicopathological parameters of 36 oral verrucous hyperplasia lesions

	Mean treatment number ± SD	*P* value*
Age		0.101
< 50 years (n = 16)	4.3 ± 1.3	
≧ 50 years (n = 20)	3.5 ± 1.5	
Location		0.446
Buccal mucosa (n = 24)	3.7 ± 1.5	
Other oral mucosal sites (n = 12)	4.1 ± 1.4	
Clinical apperance		0.000
Mass type (n = 17)	2.9 ± 1.3	
Plaque or combination type (n = 19)	4.7 ± 1.1	
Size (greatest diameter)		0.011
< 1.5 cm (n = 12)	3.0 ± 1.3	
≧ 1.5 cm (n = 24)	4.3 ± 1.4	
Color		0.000
Pink (n = 22)	3.2 ± 1.4	
White (n = 14)	4.8 ± 1.1	
Epithelial dysplasia**		0.043
With (n = 9)	3.1 ± 1.5	
Without (n = 26)	4.2 ± 1.3	
Surface keratin thickness**		0.003
≦ 40 μm (n = 23)	3.4 ± 1.4	
> 40 μm (n = 12)	4.8 ± 0.7	

*Student's *t*-test was used to compare the mean treatment numbers between any two groups.

**Biopsy was not done in one patient, because the lesion was too small.

Table 6. Comparison of clinical outcomes of 97 oral leukoplakia (OL) lesions treated with topical ALA-PDT according to lesion location and pathological diagnosis

Group of lesion	Clinical outcome		
	Complete response	Partial response	No response
21 OL lesions at the tongue	5	11	5
76 OL lesions at other oral mucosal sites	11	43	22
22 OL lesions with mild or moderate dysplasia	6	14	2
75 OL lesions without dysplasia	10	40	25

Chi-square test showed that 22 OL lesions with dysplasia were prone to have good clinical outcomes compared to 75 OL lesions without dysplasia after topical ALA-PDT treatment (marginal significance, $P = 0.052$). However, there was no significant difference in the clinical outcome between the 21 OL lesions at the tongue and 76 OL lesions at other oral mucosal sites after topical ALA-PDT treatment ($P > 0.05$).

Table 7. Comparisons of the means of surface keratin thickness, Bak/Mcl-1 LS ratio and labeling score (LS) between complete response (CR) group and partial or no response (PR or NR) group in 18 OVH and 40 OL lesions treated with topical ALA-PDT

	CR (n = 30) Mean ± SD	PR or NR (n = 28) Mean ± SD	*P* value*
Keratin thickness	41 ± 32 μm	64 ± 47 μm	0.036
Bak/Mcl-1 LS ratio	2.1 ± 1.8	0.7 ± 0.7	0.020
Bak LS	210 ± 117%	115 ± 108%	0.003
Mcl-1 LS	152 ± 97%	144 ± 91%	0.648
Caspase-3 LS	82 ± 77%	88 ± 88%	0.946
Caspase-8 LS	110 ± 107%	86 ± 98%	0.384
Caspase-9 LS	74 ± 88%	70 ± 73%	0.899
p53 LS	20 ± 24%	22 ± 43%	0.907
p21 LS	145 ± 118%	93 ± 99%	0.131
PCNA LS	136 ± 114%	137 ± 103%	0.764

*Comparison between two groups was performed by Mann-Whitney rank sum test.

Table 8. The relationship between the treatment number of cryotherapy to achieve a complete response and each of the clinicopathological parameters of 60 oral leukoplakia lesions

Clinicopathological parameters	Treatment number			Chi-square test *P* value
	1 - 6	7 - 12	13 - 17	
Age				0.701
< 50 years	16	7	3	
≧ 50 years	21	11	2	
Location				0.003
Buccal mucosa	24	9	0	
Tongue	3	4	4	
Other oral mucosal sites	10	5	1	
Area				0.024
< 2.0 cm^2	30	9	2	
≧ 2.0 cm^2	7	9	3	
Epithelial dysplasia				0.033
With	22	4	2	
Without	15	14	3	
Surface keratin thickness				0.045
< 55 μm	26	14	1	
≧ 55 μm	11	4	4	

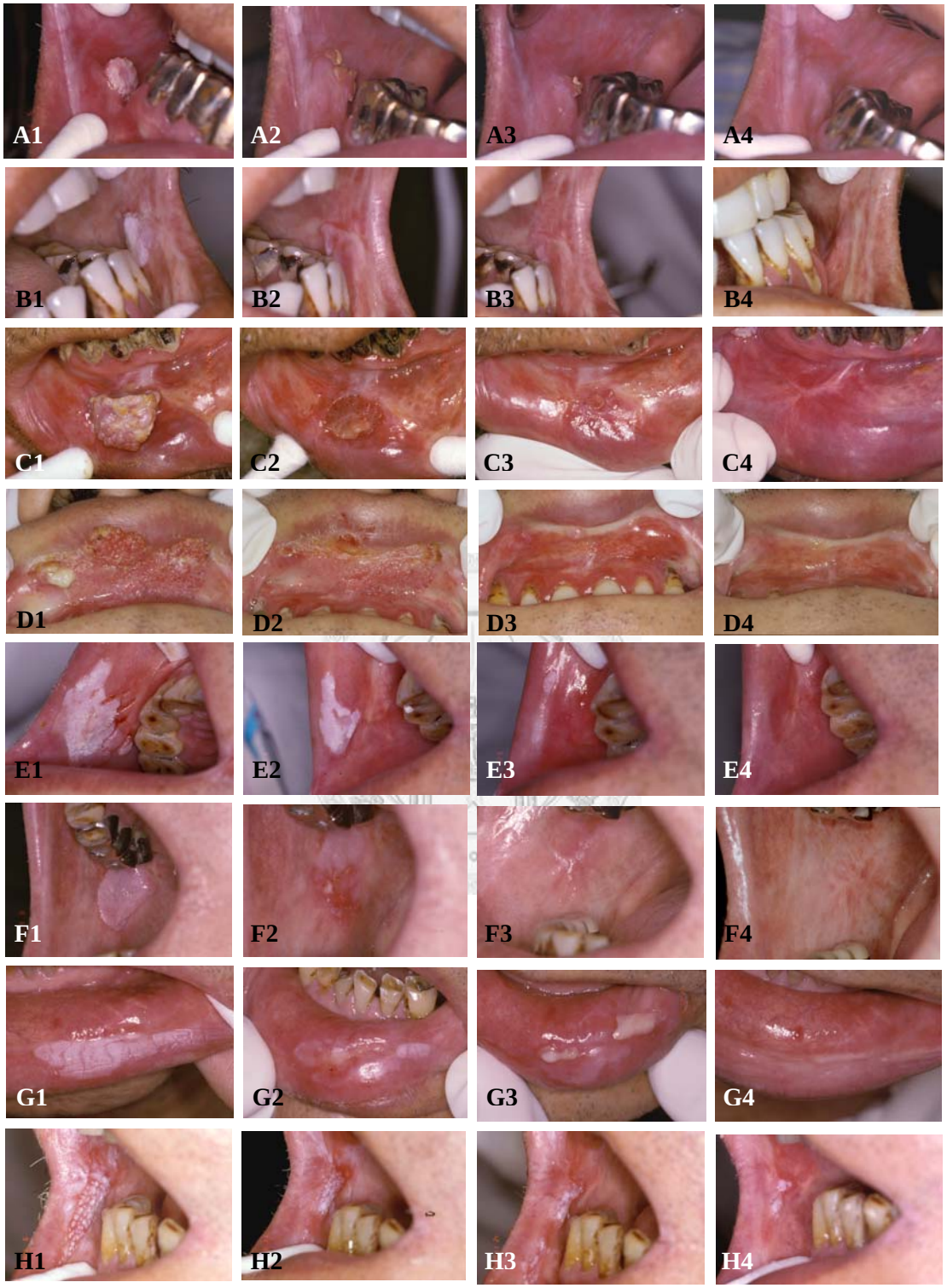
Table 9. Multivariate analyses of the treatment number of cryotherapy to achieve a complete regression and clinicopathological parameters of 60 oral leukoplakia lesions by Poisson regression model

Factor	Coefficient	*P* value
(Intercept)	1.67265	< 2e-16
Location (tongue vs. other oral mucosal sites)	0.54983	0.000176
Area (< 2.0 cm^2 vs. $\geqq 2.0$ cm^2)	0.26359	0.021280
Epithelial dysplasia (with vs. without)	-0.08352	0.482409
Surface keratin thickness (< 55 μm vs. $\geqq 55$ μm)	0.13541	0.228649

XIII. FIGURES

Figure 1. Clinical photographs of patients with oral verrucous hyperplasia (OVH) or oral leukoplakia (OL) before and after topical ALA-PDT treatment. (**A**) An OVH lesion on the right buccal mucosa before biopsy (A1), after biopsy (A2), after 1 treatment of PDT showing partial response (PR) (A3), and after 2 treatments of PDT showing complete response (CR) (A4). (**B**) An OVH lesion on the left buccal mucosa before biopsy (B1), after 2 treatments of PDT showing PR (B2), after 4 treatments of PDT showing PR (B3), and after 5 treatments of PDT showing CR (B4). (**C**) An OVH lesion on the lower labial mucosa before biopsy (C1), after 2 treatments of PDT showing PR (C2), after 3 treatments of PDT showing PR (C3), and after 5 treatments of PDT showing CR (C4). (**D**) An OVH lesion on the upper labial mucosa before biopsy (D1), after 2 treatments of PDT showing PR (D2), after 4 treatments of PDT showing PR (D3), and after 5 treatments of PDT showing CR (D4). (**E**) An OL lesion on the right mouth angle after biopsy (E1), after 1 treatment of PDT showing PR (E2), after 3 treatments of PDT showing PR (E3), and after 4 treatments of PDT showing CR (E4). (**F**) An OL lesion on the right buccal mucosa before biopsy (F1), after 2 treatments of PDT showing PR (F2), after 4 treatments of PDT showing PR (F3), and after 5 treatments of PDT showing CR (F4). (**G**) An OL lesion on the lower labial mucosa before biopsy (G1), after 2 treatments of PDT showing PR (G2), after 3 treatments of PDT showing PR (G3), and after 4 treatments of PDT showing CR (G4). (**H**) An OL lesion on the right buccal mucosa near mouth angle after biopsy (H1), after 4 treatments of PDT showing PR (H2), after 6 treatments of PDT showing PR (H3), and after 8 treatments of PDT showing PR (H4).

Figure 1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A2
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A4
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E4
F1
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F4
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Figure 2. Immunohistochemical staining for different apoptosis-associated proteins in tissue sections of oral verrucous hyperplasia (OVH) and oral leukoplakia (OL) before topical ALA-PDT. (**A**) Bak protein expresssion was evenly distributed throughout the epithelium and showed a condense immunostaining in the cytoplasm closely opposite to the cell nucleus in an OVH lesion with complete response (CR). (**B**) Nuclear Mcl-1 staining was scattered throughout the epithelium, whereas cytoplasmic Mcl-1 staining was found mainly in the lower two-thirds of the epithelium in an OL lesion with CR. (**C**) Cytoplasmic caspase-3 immunostaining was evenly distributed throughout the epithelium of an OVH lesion with CR. (**D**) Cytoplasmic caspase-8 immunostaining was found in the cytoplasm of keratinocytes of the whole layer of epithelium in an OVH lesion with CR. (**E**) Specific caspase-9 staining was found in the cytoplasm of the lower one-third epithelial cells in an OL lesion with partial response. (**F**) Expression of p53 protein was demonstrated in the nuclei of basal and parabasal epithelial cells in an OVH lesion with CR. (**G**) Nuclear p21 immunostaining was evenly scattered throughout the epithelium in an OVH lesion with CR. (**H**) Nuclear PCNA immunostaining was noted in lower two-thirds of epithelial cells in an OL lesion with CR. (Original magnification: A, B: 50×; C-H: 25×)

Figure 2

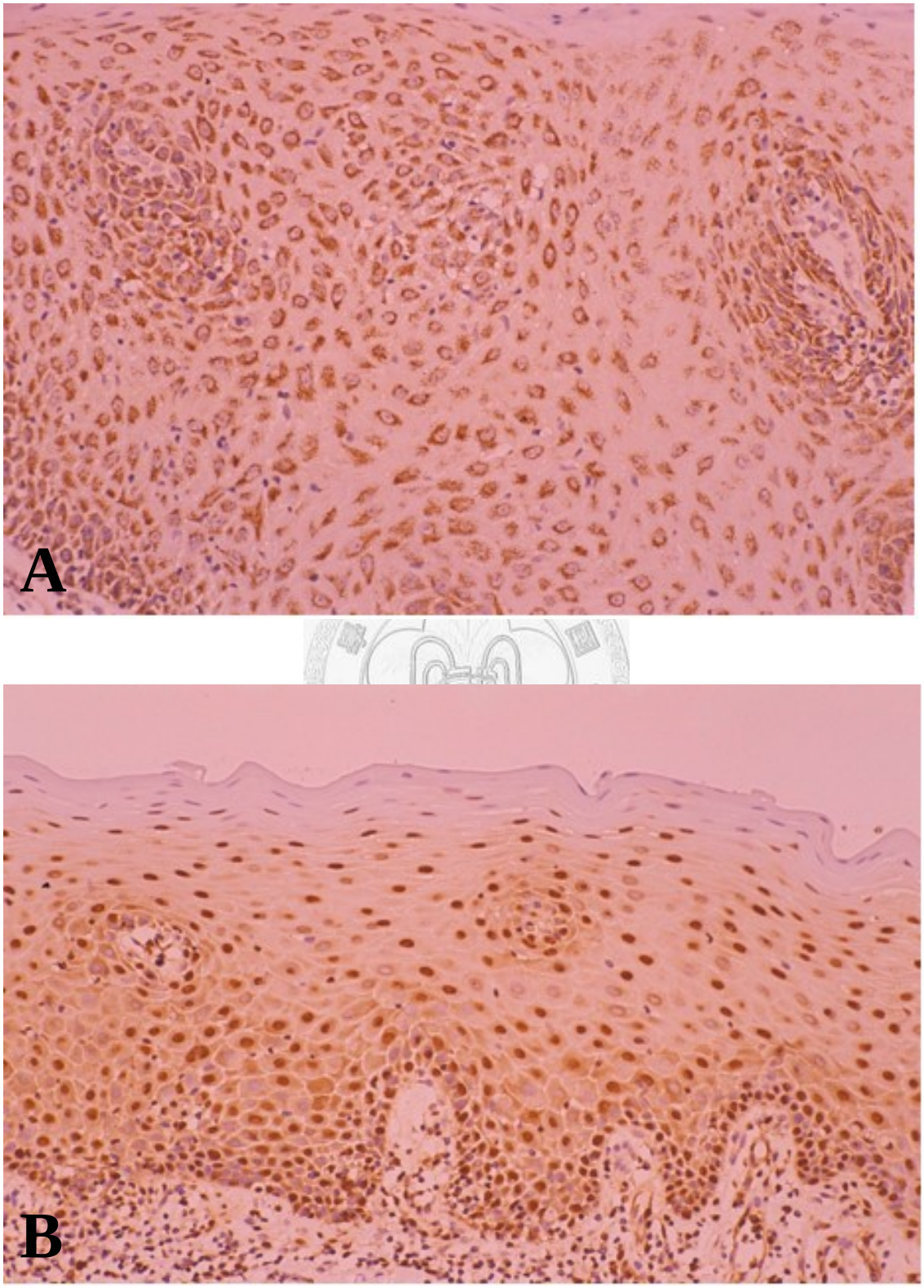


Figure 2

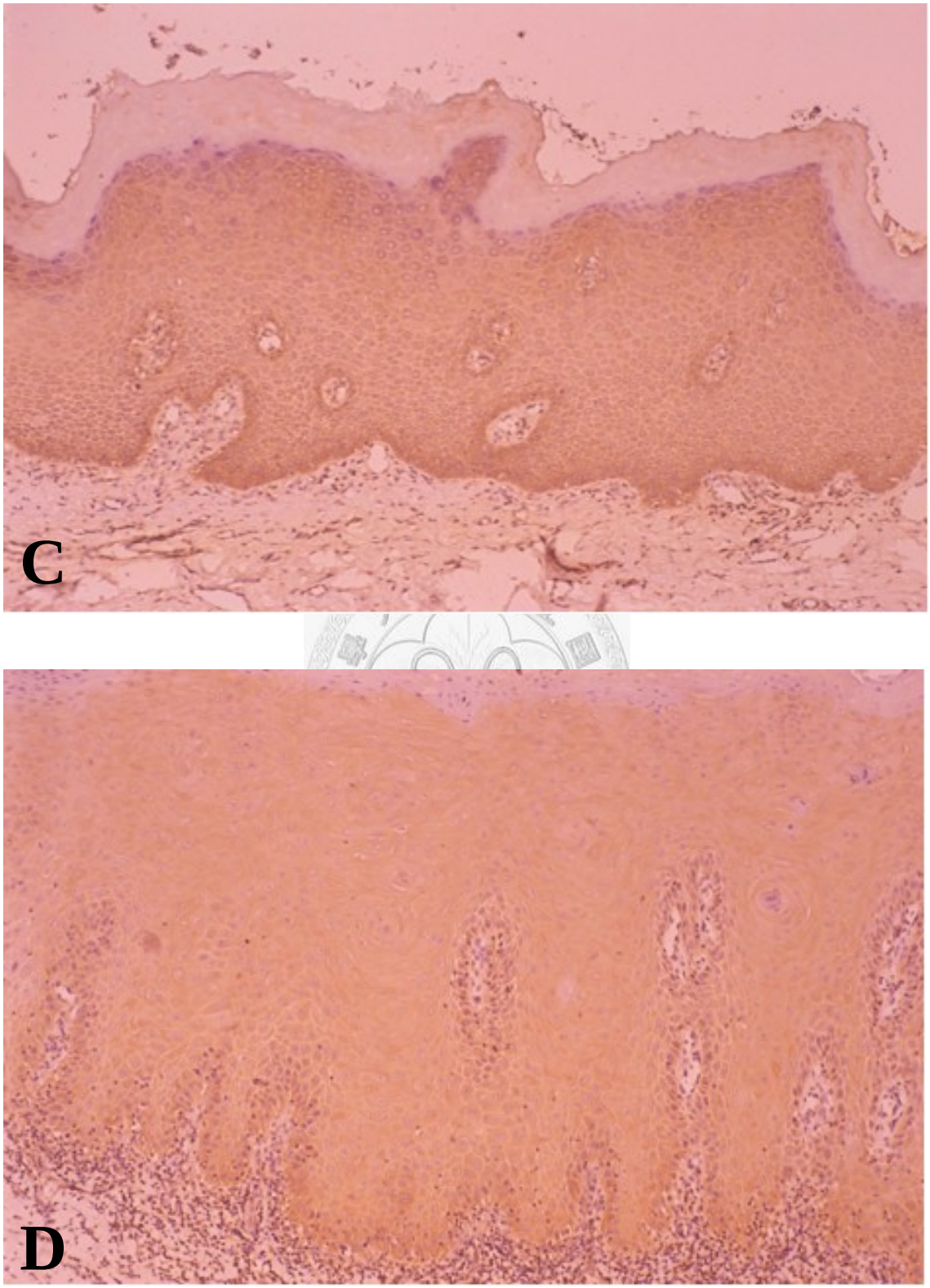


Figure 2

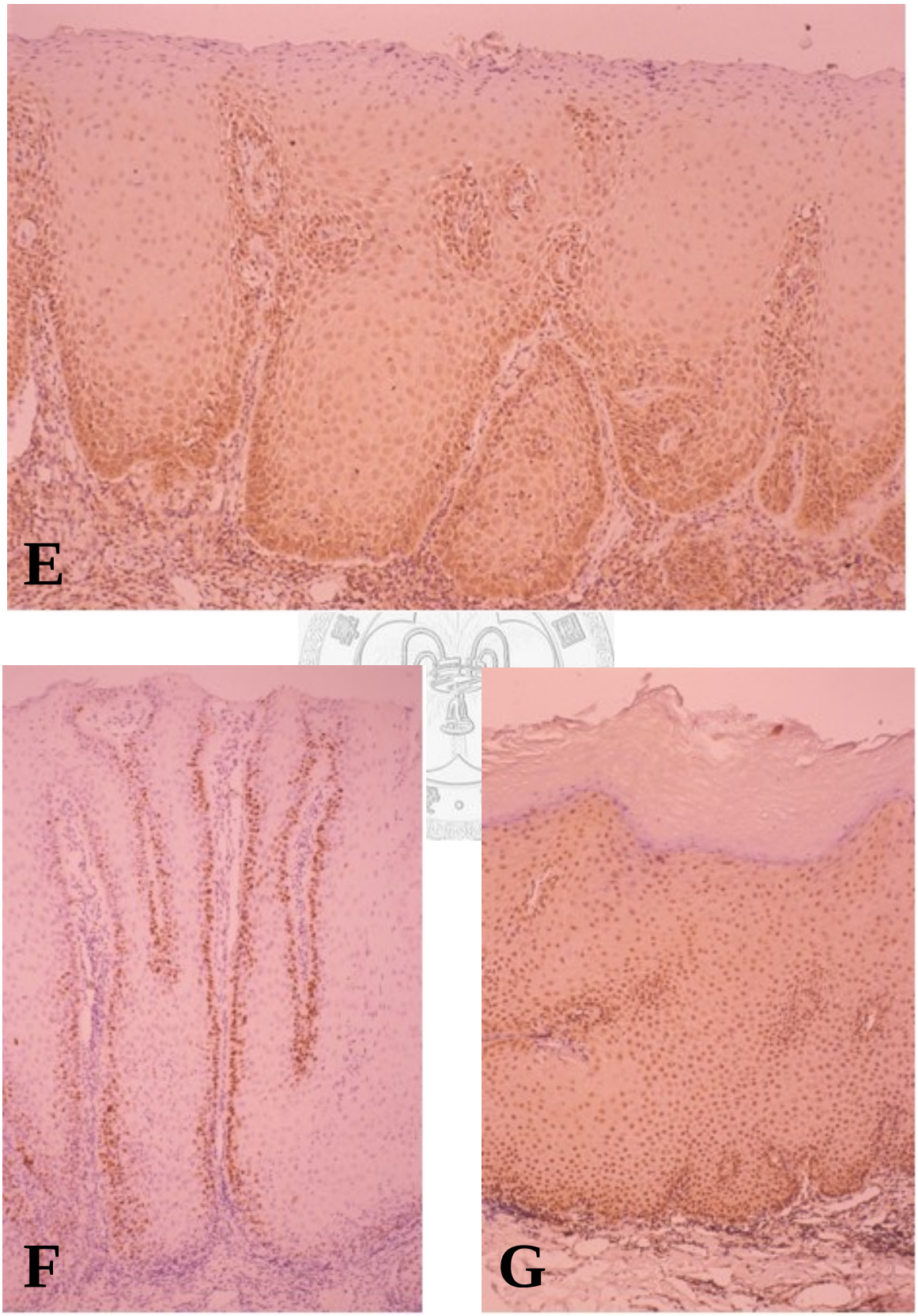


Figure 2

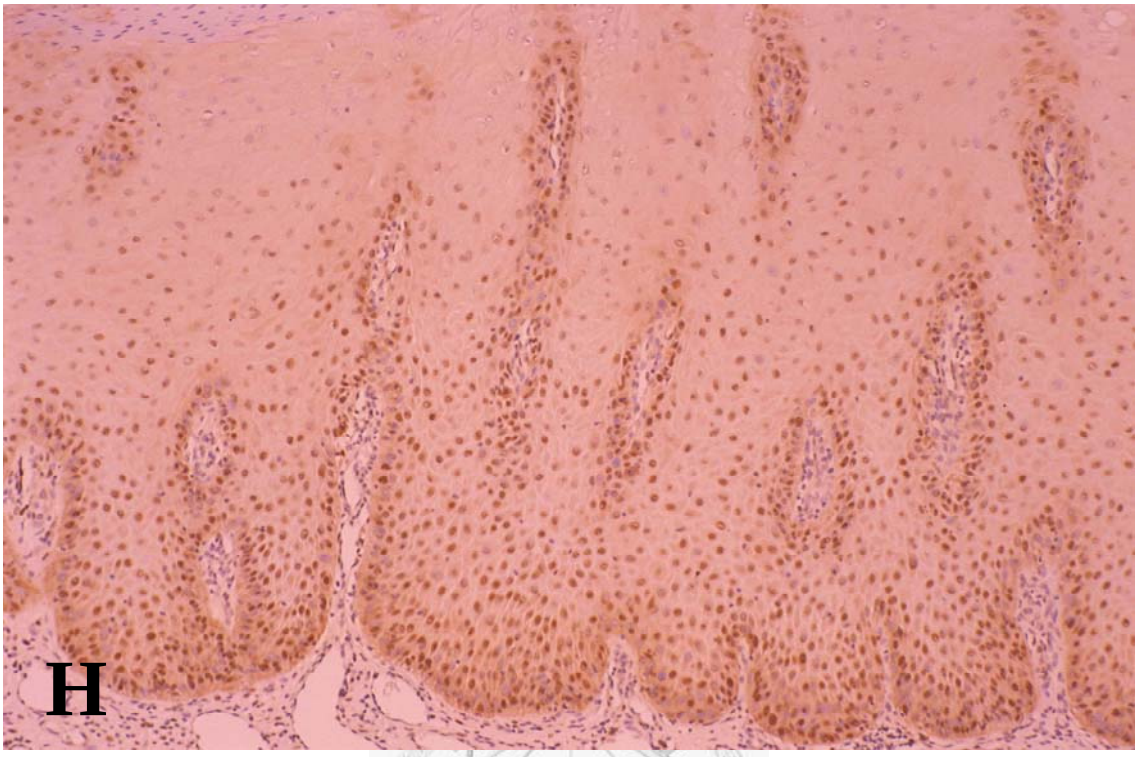
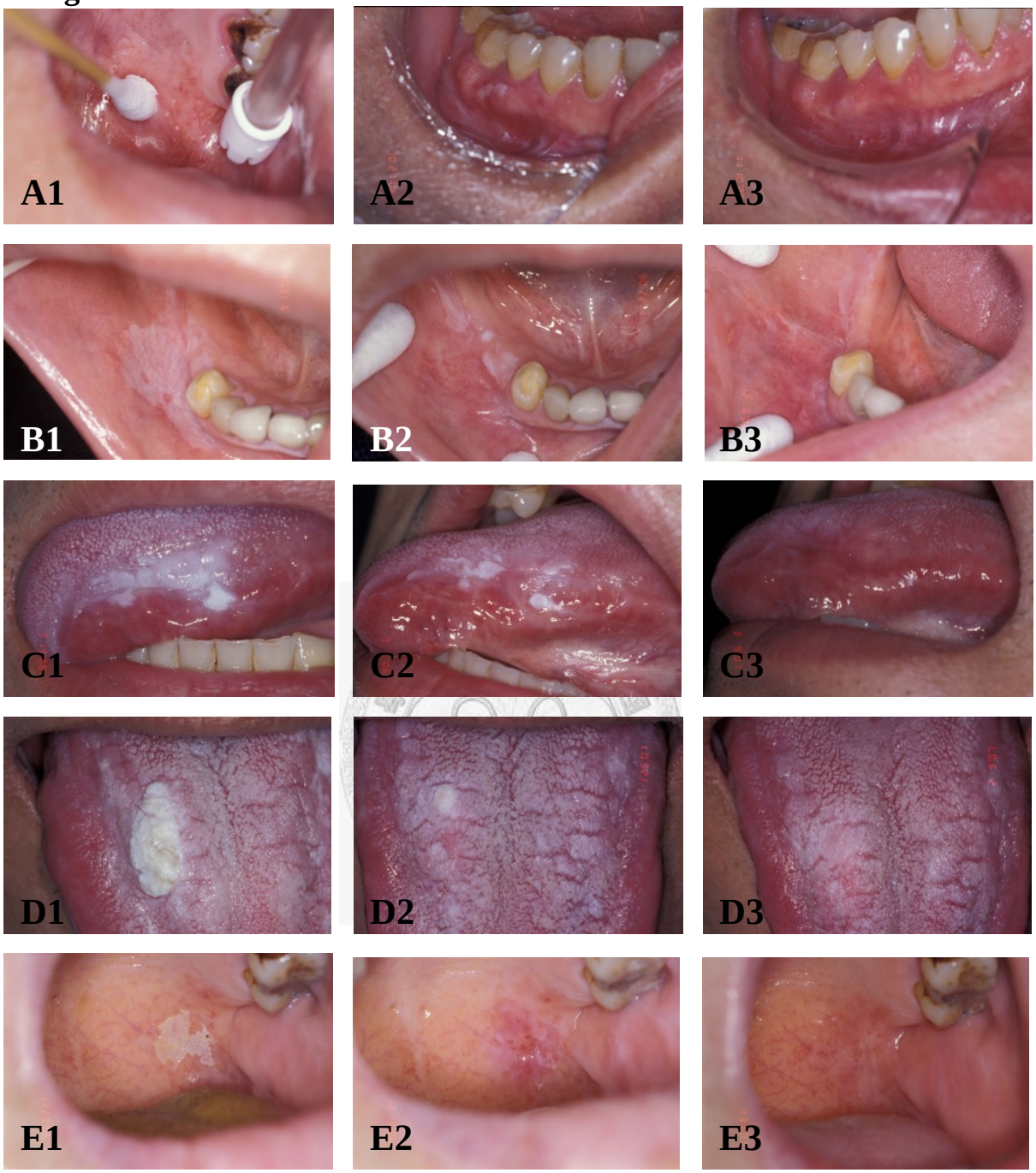


Figure 3. Clinical photographs of patients with oral leukoplakia (OL) before and after cotton-swab cryotherapy (CSC). (**A)** A cotton swab with liquid nitrogen was applied to an OL lesion on the left posterior palatal mucosa. A high-power suction was used for control of vapor fog and for an increase of visibility (A1). An OL lesion on the buccal gingiva between #45 and #46 before CSC (A2), after one treatment of CSC showing complete regression (CR) (A3). (**B)** An OL lesion on the right buccal mucosa and right lower posterior edentulous alveolar mucosa before CSC (B1), after 3 treatments of CSC showing partial regression (PR) (B2), and after 4 treatments of CSC showing CR (B3). (C) An OL lesion on the left lateral border of the tongue before CSC (C1), after 6 treatments of CSC showing PR (C2), and after 12 treatments of CSC showing CR (C3). (**D**) An OL lesion on the right dorsal surface of the tongue before CSC (D1), after 12 treatments of CSC showing PR (D2), and after 17 treatments of CSC showing CR (D3). (**E**) An OL lesion on the left posterior palatal mucosa before CSC (E1), after 3 treatments of CSC showing PR (E2), and after 5 treatments of CSC showing CR (E3).

Figure 3



XIV. APPENDIX

Curriculum Vitae

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Publications of Chuan-Hang Yu

1. Huang EYC, **Yu CH**, Cheng SJ, Chang JYF, Chen HM, Chiang CP. Decreased expression of Ep-CAM protein is significantly associated with the progression and prognosis of oral squamous cell carcinomas in Taiwan. *J Oral Pathol Med* (2008); doi: 10.1111/j.1600-0714.2008.00664.x, in press. (**SCI**)

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13. **Yu CH**, Tsai TC, Wang JT, Liu BY, Wang YP, Sun A, Chiang CP. Oral verruciform xanthoma: a clinicopathological study of 15 cases. *J Formos Med Assoc*, 2007; 106(2): 141-147. (**SCI**)

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Expression of human telomerase reverse transcriptase (hTERT) protein is significantly associated with the progression, recurrence and prognosis of oral squamous cell carcinoma in Taiwan. *Oral Oncol*, 2007; 43(2): 122-129. (**SCI**)

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