

# 行政院國家科學委員會專題研究計畫 成果報告

p21 (WAF1), Ki-67, retinoid receptors, beta-catenin  
及 E-cadherin 在眼瞼皮脂腺癌之發生機轉及預後所扮演的  
角色

研究成果報告(精簡版)

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Topic: The role of p21 (WAF1), Ki-67, retinoid receptors, beta-catenin and E-cadherin in the pathogenesis and prognosis of eyelid sebaceous gland carcinoma

題目: p21 (WAF1), Ki-67, retinoid receptors, beta-catenin 及 E-cadherin 在眼瞼皮脂腺癌之發生機轉及預後所扮演的角色

## Abstract

**Purpose:** to study the correlation of expression of  $\beta$ -Catenin, E-cadherin, Ki-67 markers, RAR- $\beta$ , RAR- $\gamma$  and RXR- $\beta$  in sebaceous glands carcinoma and tumor metastasis.

**Methods:** Immunohistochemistry of  $\beta$ -Catenin, E-cadherin, Ki-67 markers, RAR- $\beta$ , RAR- $\gamma$  and RXR- $\beta$  from pathological specimens of sebaceous gland carcinoma. Metastasis information was collected from the chart reviews or telephone interview. The correlation of the expression of different markers and tumor metastasis was measured by Fisher's exact test.

**Results:** Total 23 pathological specimens of sebaceous gland carcinoma were collected. Among the 23 patients, 2 patients lost of follow up and 4 patients was found to have metastasis. We found that more tumor cells in the tumor part were stained by  $\beta$ -Catenin, E-cadherin and Ki-67 markers than cells in normal tissues. On the contrary, more cells in the normal tissues were stained by retinoid receptors markers than tumor cells in the tumor part. And there was no statistically significant correlation between tumor metastasis and the expression of different markers.

**Conclusion:** From the long term clinical follow up and the expression of  $\beta$ -Catenin, E-cadherin, Ki-67, RAR- $\beta$ , RAR- $\gamma$  and RXR- $\beta$  IHC stain from sebaceous gland carcinoma, we found that there seemed no correlation between tumor metastasis and the expression of those markers.

## **Background and Purpose**

Sebaceous gland carcinoma is a highly malignant and potentially lethal tumor arising from meibomian, Zeis, or sebaceous glands of the skin. It occurs most frequently in the upper lids of females over age 50 [1,2]. Earlier definitive treatment including wide surgical excision or even orbital exenteration improves prognosis [2] and yields much lower mortality rates [1,3,4,5].

It usually metastasizes to regional lymph nodes but may also spread hematogenously or through direct extension. Regional lymph node or distance metastasis occurs in 10-20% of patients [6]. In recent years, some researches tried to identify the biomarkers that may be important in the metastasis and prognosis of malignant tumors including sebaceous carcinoma.

In view of proliferation, p21 (WAF1), a nuclear protein that regulates cell cycle progression, can be transcriptionally upregulated by p53, but may be activated independently of p53-for example, during terminal differentiation. Loss of topological control of p21 (WAF1) expression is in sebaceous carcinoma and adenomas at an early stage of malignancy in the colorectal system [7].

Ki-67 nuclear antigen is expressed in all phases of the cell cycle (G1,S,G2, and M), but is undetectable in G0. So it is regarded as the most reliable indicators of cellular proliferation. The labeling index of Ki-67 is significantly higher in sebaceous carcinoma than in squamous cell carcinoma, conjunctival intraepithelial neoplasia, pterygium and normal conjunctiva [8].

The retinoids, including vitamin A, have been shown to affect the normal growth and differentiation of epithelial tissue [9]. It is thought that the biologic actions of retinoids are mainly mediated by nuclear retinoid receptors. There are two classes of retinoid receptors, RARs and RXRs, each with three subtypes, alpha, beta, and gamma. Retinoids have been shown to suppress oral, skin, bladder, lung, prostate, and breast carcinogenesis in experimental animals [10,11,12]. Expression of retinoid receptors are decreased or absent in sebaceous cell carcinoma of the eyelid compared with controls [13].

In cell-to-cell adhesion, E-cadherin links to internal cytoskeleton by catenins (alpha, beta, and gamma). Reduced expression of the catenins and E-cadherin has been associated with a worse prognosis in patients with NSCLC [14,15].

## **Methods**

### ***Patients and Tumor Samples***

Paraffin embedded sections of eyelid sebaceous carcinoma will be selected by consulting the archives of the Department of Pathology, National Taiwan University Hospital. Chart reviews and telephone interviews of patients will be performed to collect the clinical data such as recurrence, metastasis, mortality, and association with visceral malignancy, etc. Paraffin embedded sections of eyelid sebaceous carcinoma will be processed for following immunohistochemistry and DNA sequence study.

### ***Immunohistochemistry of biomarkers***

#### **1. Immunohistochemistry of p21WAF1 and p53**

Sections (5  $\mu$  m thick) of paraffin wax embedded tissue are dewaxed in xylene, rehydrated, and microwave enhanced antigen retrieval is performed in Trilogy (Cell Marque).

Immunohistochemistry is performed using mouse monoclonal antibodies to either p21WAF1 (1/50 dilution; Oncogene Research Products, Cambridge, Massachusetts, USA) and p53 (1/50 dilution; DO7; Novocastra Laboratories, Newcastle, UK). Immunoperoxidase staining using diaminobenzidine as a chromogen is run with the NEX-ES Automatic Staining System (Ventana, Strasbourg, France). Sections are counterstained with haematoxylin.

#### **2. Immunohistochemistry of Ki-67**

All formalin-fixed and paraffin-embedded sections about 5  $\mu$  m thick are cut for Ki-67 antibody immunostaining. First, paraffin sections are incubated in a 55 °C oven for 30 min. The sections are then dewaxed in xylene and rehydrated in graded ethanol, immersed in Trilogy (Cell Marque), and heated in an autoclave for 30 mins for

antigen retrieval. The sections are incubated at room temperature for 2 h with Ki-67 antibody (MIB-I, 1:50; Dako, Glostrup, Denmark). After three further washings with PBS milk, immunoperoxidase staining using diaminobenzidine as a chromogen is run with the NEX-ES Automatic Staining System (Ventana, Strasbourg, France), and counterstained with hematoxylin. The number of Ki-67-labeled nuclei in tumor cells is counted using a 10×10 square grid graticule at ×400 magnification. The areas for cell counting are selected from the most mitotically active parts of the tumors. The sections are scanned at low magnification for the most densely and evenly labeled areas. Unequivocal nuclear staining is considered a positive reaction, regardless of staining intensity. The immunoreactive score, evaluated by counting tumor cells using a 10×10 square grid graticule in at least five consecutive fields at high power (×400), is expressed as a percentage of the total cell count.

### 3. Immunohistochemistry of retinoid receptors

Serial sections (5  $\mu$  m thick) are deparaffinized in xylene and hydrated in graded ethanol, and antigens are retrieved by boiling the sections in Trilogy (Cell Marque), for 30 minutes. The sections are then incubated overnight at 4°C with the following affinity-purified rabbit polyclonal antibodies against RAR  $\alpha, \beta$  and  $\gamma$ , and RXR  $\alpha, \beta$  and  $\gamma$  of human origin, all at 1:1000 dilution (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.).

Subsequently, the sections are washed in PBS and immunoperoxidase staining using diaminobenzidine as a chromogen is run with the NEX-ES Automatic Staining System (Ventana, Strasbourg, France), counterstained with hematoxylin, and mounted for evaluation. For negative control of immunoreactions, the primary antibodies are replaced with preimmune rabbit serum according to the first antibody.

To establish the staining pattern for RAR and RXR receptors, nuclear staining is classified as follows according to the percentage of cells stained: 0, 30% up to 50%; and 3, >50% and up to 100%. The cytoplasmic and membranous staining is graded according to intensity on a scale of 0 to 3, with 0 indicating no cytoplasmic staining

and 3 indicating strong staining.

#### 4. Immunohistochemistry for $\beta$ -Catenin Protein

The 5  $\mu$ m sections of formalin fixed, paraffin embedded tissues are deparaffinized. They are treated with an autoclave in Trilogy (Cell Marque) for 30 minutes to retrieve the antigenicity. The sections then are consecutively reacted with a mouse antihuman  $\beta$ -catenin monoclonal antibody (clone 14; Transduction Laboratories, Lexington, KY) at the dilution of 1:300 at 4 °C overnight. Immunoperoxidase staining using diaminobenzidine as a chromogen is run with the NEX-ES Automatic Staining System (Ventana, Strasbourg, France), counterstained with hematoxylin. The specificity of the immunostaining is confirmed by negative control staining using mouse nonimmune immunoglobulin G instead of the primary antibody.

#### 5. Immunohistochemistry for E-cadherin Protein

The 5  $\mu$ m sections of formalin fixed, paraffin embedded tissues are deparaffinized. They are treated with an autoclave in Trilogy (Cell Marque) for 30 minutes to retrieve the antigenicity. The sections then are consecutively reacted with a mouse antihuman  $\beta$ -catenin monoclonal antibody (clone 14; Transduction Laboratories, Lexington, KY) at the dilution of 1:300 at 4 °C overnight. Immunoperoxidase staining using diaminobenzidine as a chromogen is run with the NEX-ES Automatic Staining System (Ventana, Strasbourg, France), counterstained with hematoxylin. The specificity of the immunostaining is confirmed by negative control staining using mouse nonimmune immunoglobulin G instead of the primary antibody.

### **Evaluation of Immunohistochemistry and statistics**

Immunohistochemistry of E-cadherin Protein, p53, RAR $\alpha$ ,  $\beta$ ,  $\gamma$ , RXR $\alpha$  and  $\gamma$  was evaluated by the density of stain of tumor section as compared to normal tissue section. Immunohistochemistry of Ki-67 and RXR $\beta$  was measured by the percentage of stain in the specimen section. Statistics were measured by student t test or Fisher exact test.

## **Result**

Total 23 pathological specimens of sebaceous gland carcinoma were collected from Department of pathology in national Taiwan University Hospital. Among the 23 patients, 2 patients lost of follow up and 4 patients was found to have metastasis. And the IHC expression of each marker was shown in Table 1. We found that more tumor cells in the tumor part were stained by  $\beta$ -Catenin, E-cadherin and Ki-67 markers than cells in normal tissues. On the contrary, more cells in the normal tissues were stained by retinoid receptors markers than tumor cells in the tumor part.

The correlation of sebaceous gland carcinoma metastasis with the IHC expression of different markers was shown in Table 2-7. There was no statistically significant correlation between tumor metastasis and the expression of different markers.

## **Conclusion**

From the long term clinical follow up and the expression of  $\beta$ -Catenin, E-cadherin, Ki-67 markers, RAR- $\beta$ , RAR- $\gamma$  and RXR- $\beta$  in IHC stain of pathological specimens from sebaceous gland carcinoma, we found that there seemed no correlation between tumor metastasis and the IHC expression of those markers.



Table 1 Different IHC stain pattern in specimens of sebaceous gland carcinoma

IHC Item	Number of IHC stains		Total Number
	Tumor>normal	Normal $\geq$ Tumor	
$\beta$ -Catenin	17 (74%)	6 (26%)	23
E-cadherin	10 (66.7%)	5 (33.3%)	15
Ki-67	12 (57%)	9 (43%)	21
RAR- $\beta$	6 (26%)	17 (74%)	23
RAR- $\gamma$	9 (39%)	14 (61%)	23
RXR- $\beta$	6 (26%)	17 (74%)	23

Table 2 Correlation of sebaceous gland carcinoma metastasis with  $\beta$ -Catenin IHC stain pattern

		$\beta$ -Catenin		Total
		Tumor>Normal	Normal $\geq$ Tumor	
Metastasis	No	12	5	17
	Yes	4	0	4
Total		16	5	21

Fisher's Exact test: P=0.532

Table 3 Correlation of sebaceous gland carcinoma metastasis with E-cadherin IHC stain pattern

		E-cadherin		Total
		Tumor>Normal	Normal $\geq$ Tumor	
Metastasis	No	8	4	12
	Yes	0	1	1
Total		8	5	13

Table 4 Correlation of sebaceous gland carcinoma metastasis with Ki-67 IHC stain pattern

		Ki-67		Total
		Tumor>Normal	Normal $\geq$ Tumor	
Metastasis	No	9	6	15
	Yes	3	1	4
Total		12	7	19

Fisher's Exact test: P=1

Table 5 Correlation of sebaceous gland carcinoma metastasis with RAR- $\beta$  IHC stain pattern

		RAR- $\beta$		Total
		Negative	Positive	
Metastasis	No	11	6	17
	Yes	4	0	4
Total		15	6	21

Fisher's Exact test: P=0.281

Table 6 Correlation of sebaceous gland carcinoma metastasis with RAR- $\gamma$  IHC stain pattern

		E-cadherin		Total
		Negative I	Positive	
Metastasis	No	6	11	17
	Yes	3	1	4
Total		9	12	21

Fisher's Exact test: P=0.272

Table 7 Correlation of sebaceous gland carcinoma metastasis with RXR- $\beta$  IHC stain pattern

		RXR- $\beta$		Total
		Tumor>Normal	Normal $\geq$ Tumor	
Metastasis	No	3	14	17
	Yes	2	2	4
Total		5	16	21

Fisher's Exact test: P=0.228

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政院國家科學委員會補助國內專家學者出席國際學術會議報告

2006年12月24日

報告人姓名	廖述朗醫師	服務機構及職稱	臺大醫院眼科部 主治醫師
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發表演文題目	(中文) 利用眼窩抽脂手術處理甲狀腺突眼的結果及預測性 (英文) Results and predictability of fat removal orbital decompression for disfiguring dysthyroid exophthalmos		

## 一、參加會議經過

美國眼科醫學會及眼部整形及顏面重建會議，為全世界眼科、眼部整形及顏面重建相關領域學術研討最重要的會議。每年舉辦一次，在美國不同的城市輪流舉辦，是眼科一年一度的盛會。有來自全世界眼科相關領域的醫師來參與，並提出最新的研究論文、手術技術及儀器，是一個吸收新知並提昇台灣眼科及眼部整形顏面重建的知名度的很好機會。

這次美國眼科醫學會及眼部整形及顏面重建學術研討會，在拉斯維加斯舉辦，本人提出一篇兩百多個病例的研究論文，報告本院眼科對於甲狀腺眼疾造成嚴重突眼的病患，我們使用眼窩抽脂減壓手術（Fat Removal Orbital Decompression）治療的效果，並利用迴歸方程式計算每取一毫升脂肪可以達到多少眼球後縮的效果，用此一方程式可以預測減壓手術的效果。由於這是一種較新且可以有效並迅速地解決病患突眼問題的手術，在會中我們提出手術治療的成果，並獲得與會醫師很多的迴響。

## 二、與會心得

本次會議的眾多報告（presentations）及討論中，有很多新的觀念及值得眼科進一步研究及發展的方向，分述如下：

1. 使用分子生物學的方法（molecular biology）來研究黑色素瘤（melanoma）：利用基因 Microarray analysis 來篩檢出一些表現較高的 mRNA 如 Cadherin-3, NCOA3, CDH3 等，再利用 real time PCR, IHC stain 及其他 protein analysis 的方法



來分析這些因子和黑色素瘤的轉移是否有相關。這研究可提供黑色素瘤病患判斷術後轉移及預後的依據。這是相當先進的研究，也提供我們眼科基礎研究的新方向。

2. 數位紅外線體溫測量圖(digital infrared thermography)來評估甲狀腺眼病變的治療成效：這次會議提出相當多的論文，其中較具啟發性的是談到利用數位紅外線體溫測量的儀器來測量顏面體表的溫度。由於顏面體表的溫度和發炎程度有正相關，可以用這一種體溫測量圖來評估甲狀腺眼病變藥物治療後的成效。這是一個很好的創見，或許我們也可以利用紅外線體溫測量的儀器來對眼窩免疫性發炎或蜂窩性組織炎做鑑別診斷，並提供治療的方針。這也是台灣眼科可以進一步發展的診斷技術。另外，對於眼部周圍的血管瘤在這次的研討會中也有一個專題，詳細地討論這類疾病的各種治療方式及長期預後。有了這些資訊，相信台灣血管瘤病患一定可以得到更完善的照顧。

總之，醫療科技的進步日新月異，加上E化資訊傳遞的迅速，每次參加這類世界性的國際會議，總是有很多新穎的技術和觀念，我們眼科部也戰戰兢兢地學習和創新且積極地研究發展，並進一步發表於國際學術會議上。對於上面提到本次研討會值得注意的觀念及研究，我們也將列入我們研究發展的重點，希冀很快地就能迎頭趕上，並發表論文於相關國際期刊，如此才能不愧對貴會的支持及鼓勵！

### 三、建議

雖然現在網路傳遞訊息相當快速便捷，很多相關期刊資料可以從網路截取。然而，參加知名的國際性會議，一方面可吸取最新、最即時的研究新知，還可以藉由和知名學者的討論，從中嗅到最新的眼科發展趨勢，並非網路資訊所能取代。因此建議貴會應多鼓勵年輕醫師出國參加研討會並發表研究論文。

#### 四、攜回資料名稱及內容

會議手冊及各報告題目摘要(如附件)