行政院國家科學委員會專題研究計畫 成果報告

碳酸酐異構酶 IX, XII 在口腔黏膜下纖維化及口腔癌的表現(第 3 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 96-2628-B-040-021-MY3
執 行 期 間 ：98 年 08 月 01 日至 99 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學研究所

計 畫 主 持 人：楊順發
共 同 主 持 人：謝易修、張育超
計畫參與人員：博士班研究生-兼任助理人員：楊嘉欣

報 告 附 件 ：出席國際會議研究心得報告及發表論文

處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 99 年 10 月 29 日

Alternative splicing variants of carbonic anhydrase IX in oral cancer

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Keywords:

Oral cancer, Hypoxia; CAIX; FL; AS; Real-time RT-PCR

Abbreviations:

OSCC, Oral squamous cell carcinoma; CAIX, carbonic anhydrase IX; FL, full length; AS, alternative splicing; RT-PCR, Reverse-transcriptase polymerase chain reaction.

Abstract

Objectives: Carbonic anhydrase IX (CAIX) is a zinc metalloenzyme, which contributes to maintain intracellular and extracellular pH under hypoxic conditions. Alternative splicing of the *CA9* transcript generates two isoforms. One is full-length (FL) mRNA, which composed of all eleven exons that encodes a plasma membrane-localized and functionally competent CA IX protein. Another is alternative splicing (AS) mRNA, which lack the exons 8 and 9 that encode the transmembrane region, the intracellular tail and the C-terminal of the catalytic domain. However, the levels of FL and AS CAIX between normoxia and hypoxia in oral cancer were still unclear. **Methods**: Six oral cancer cell lines were subjected to a treatment with $CoCl_2$ or hypoxia. Furthermore, reverse-transcription PCR and real-time PCR were performed to investigate the mRNA expression of FL and AS CAIX isoforms in oral cancer cell lines. **Results:** The mRNA expression of FL and AS CAIX were significantly higher in oral cancer cells, which treated with $CoCl_2$ or hypoxia than normoxia ($P<0.001$). **Conclusion:** The data presented here demonstrated that FL and AS CAIX mRNA increased when cells underwent hypoxic conditions. These results demonstrated that FL and AS CAIX might be contributed to the development of oral cancer under hypoxic conditions.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity with a poor prognosis, which is closely related to cancer progression. Usually, cancer progression is accompanied by deep alterations of microenvironmental conditions in which tumor cells proliferate, including the progressive reduction of oxygen supply. Hypoxia is a common event in locally advanced solid tumors, frequently associated to cancer progression, genetic instability, selection for resistance to apoptosis, increased risk of invasion and metastasis, poor response to radiation and chemotherapy [Brizel et al, 1996; Graeber et al, 1996; Hockel et al, 1996; Reynolds et al, 1996; Kim et al, 1997]. Hypoxia results in the upregulation of genes that facilitate anaerobic metabolism and promote tumor vascularisation. The transcriptional complex hypoxia-inducible factor-1 (HIF-1) is the major mediator of gene expression in hypoxic cancer cells. [Sly and Hu, 1995]. Strong induction by HIF, hypoxia-related intratumoral distribution, and the relationship to cancer development and/or treatment outcome predispose CA IX to serve as a surrogate marker of hypoxia with a prognostic value [Wykoff et al, 2000; Beasley et al, 2001; Potter and Harris, 2003; Watson et al, 2003].

CAIX is a transmembrane glycoprotein, member of a family of zinc metalloenzymes that reversibly converts carbon dioxide and water to bicarbonate and

proton, and thereby contribute to modulation of ion transport and maintenance of acid-base balance. CAIX plays a relevant role in pH regulation and its expression reduces the pericellular pH, facilitating breakdown of the extracellular matrix [Giatromanolaki et al, 2001]. This leads to intracellular alkalosis and extracellular acidosis in the tumor microenvironment, allowing to tumor cells to survive under hypoxic conditions [Stubbs et al, 2000; Thiry et al, 2006]. While most CA isoforms are uniformly expressed in differentiated cells of the normal tissues, CAIX is predominant in cancer cells [Potter and Harris, 2003]. The expression of CAIX has been correlated with clinical outcome in several human cancers [Chia et al, 2001; Loncaster et al, 2001; Kaanders et al, 2002; Swinson et al, 2003; Hussain et al, 2004].

Very recently, besides the expected FL mRNA, a CAIX alternative splicing isoform was detected in normal and in cancer cells. This alternative splicing (AS) generates a transcript lacking of exons 8-9 and encodes a truncated CAIX protein lacking the transmembrane region, the intracellular tail and the C-terminal part of the catalytic domain. AS CAIX shows a diminished catalytic activity that reduces the capacity of the full-length CAIX protein to acidify extracellular pH of hypoxic cells and to bind carbonic anhydrase inhibitor [Barathova et al, 2008]. However, the levels of FL and AS CAIX between normoxia and hypoxia in oral cancer were still unclear. In the present study we measured the mRNA expression of FL and AS CAIX

isoforms in six oral cancer cells to explore their independent manifestation.

2. Materials and methods

2.1 Cell and cell culture

SCC-4 and SCC-9, human tongue SCC cells obtained from ATCC (Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with a nutrient mixture, F-12 Ham's medium (Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 400 ng/mL hydrocortisone. Human SCC cell lines, HSC3 and SAS derived from tongue carcinoma, as well as OC2 cell line from human buccal mucosa cancer were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. OECM-1, a human Gingival carcinoma, was cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Hypoxic treatments were performed in an anaerobic workstation (Ruskin Technologies, Bridgend, UK) in 2% O_2, 5% CO_2, and 93% N_2 at 37°C. All cell lines allowed to adhere for 24 h and grow for additional 24 h in normoxia and parallel in hypoxia or 100μM $CoCl_2$.

2.2 Reverse-transcriptase PCR

Total RNA was isolated from cells using an RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the concentration of total RNA was measured spectrophotometrically. Reverse transcription was performed with M-MuLV reverse transcriptase (Finnzymes, Oy, Finland) using random heptameric primers (400 ng/μl). The mixture of 5 mg of total RNA and random primers (400 ng/μl) was heated for 10 min at 70°C, cooled quickly on ice and supplemented with 0.5 mM dNTPs (Finnzymes), reverse transcriptase buffer containing 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 0.1 mg/ml BSA, and 50 mM Tris-HCl, pH 8.3. The mixture in a final volume of 24 ml was further supplemented with 200 U of reverse transcriptase M-MuLV, incubated for 1 h at 42°C, heated for 15 min at 70°C, and stored at -80°C until used.

PCR was performed with Dynazyme EXT polymerase (Finnzymes) with the primers listed in Table 1. The protocol of PCR consisted of 94°C for 5 min followed by 18-35 cycles of denaturing at 94°C for 1 min, annealing for 1 min (temperature depended on sets of primers), and extension at 72°C for 2 min, followed by a final extension at 72°C for 20 min. Resulting PCR fragments of each sample was analyzed by electrophoresis in a 2% agarose gels, and visualized by ethidium bromide staining. The amount of PCR products was semiquantitatively expressed as the ratio of the

intensity of each band to the intensity of the related GAPDH internal standard.

2.3 Real-time PCR

The cDNA was obtained as described above. Real-time PCR was performed with TaqMan® Gene Expression Master Mix (Applied Biosystems) with primers specific for FL (h8A) and AS (h10/7A), respectively, combined with h7S primer common for both transcripts. Real-time PCR was run on Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems), using the following program, UDG incubation at 50°C for 2 min, initial denaturation at 95°C for 10 min followed by cycling (40 cycles) denaturation at 95°C for 15 s, annealing at 60°C for 60 s. All PCRs were performed in duplicates and repeated three times. The amount of each type of PCR product was normalised against GAPDH and the ratio between FL and AS was calculated for cell lines.

2.4. Western blot analysis

For total cell lysates preparation, cells were rinsed with PBS twice and scraped with 0.2 mL of cold RIPA buffer containing protease inhibitors cocktail, and then vortexed at 0°C for 20 min. Cell lysates were subjected to a centrifugation of

10,000×*g* for 20 min at 4°C, and the insoluble pellet was discarded. The protein concentration of total cell lysates was determined by Bradford assay.

The cell lysates were separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The blot was subsequently incubated with 5% non-fat milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 hours to block non-specific binding, and probed with CAIX (Santa Cruz Biotechnology, CA, USA) and β-actin (Biosource, Camarillo, CA). Blots were detected with appropriate peroxidase conjugated secondary antibody for 1 hour. Afterwards, signal was measured by using enhanced chemiluminescence (ECL) commercial kit (Amersham Biosciences) and relative photographic density was quantitated by analysis system (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

2.5. Wound healing assay

HSC3 cells were seeded in 60-mm dishes and grown to 90% confluence, and then starvation with DMEM media with 0.5% FBS for 16 h. The monolayer of cells was scratched with a sterile 200 μL pipette tip to create a wound, and the plates were washed twice with PBS to remove floating cells, and then DMEM with 0.5% FBS media was placed in the plates. Cells were subjected to the indicated for 24 h in

normoxia and parallel in hypoxia, and cells migrating from the leading edge were photographed at 0, 12 and 24 h. The data presented are representative of 3 independent experiments.

2.6. Statistical Analysis

Values were expressed as the mean ± S.D. Statistical significances of difference throughout this study were calculated by Student's t-test (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA). A difference at $p<0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of human CA IX in oral cancer cell lines

To determine the expression of CAIX in six oral cancer cell lines, the mRNA levels were analyzed by RT-PCR with h1S-h6A primers that do not discriminate between the splicing variants (Fig. 1A). Furthermore, the protein levels of CAIX were analyzed by Western blot (Fig. 1B).

3.2. Hypoxia and $CoCl_2$ induce CA IX expression in human oral cancer cell lines

In order to elucidate the effects of hypoxic stress conditions on the expression of CAIX, six oral cancer cell lines were incubated under hypoxic conditions (2% O_2) or

stimulated by a biochemical hypoxia mimetic ($CoCl_2$). Both hypoxic conditions and $CoCl_2$ induced an increase in the level of CAIX mRNA (Fig. 2A) and protein (Fig. 2B).

3.3. Expression of FL-CA IX and AS-CA IX in human oral cancer cell lines.

Since it was previously reported that AS-CAIX does not contain the exons 8 and 9, we used the forward primer located on exon 7 (h7S), which is common for both the transcripts, in combination with primer h8A, present only on FL variant. Conversely, we amplified the AS fragment from a PCR with h7S and h10/7A primers. From these separated PCRs we obtained the two expected amplification products: for FL mRNA the length was 154 bp and 140 bp for AS mRNA variant, respectively (Fig. 3).

3.4. Hypoxia and $CoCl_2$ induce FL-CA IX and AS-CA IX expression in human oral cancer cell lines

We analyzed the presence of the variants in the human oral cancer cell lines exposed to normoxia (20% O_2) and hypoxia (2% O_2). The mRNA of FL and AS variants was detected in all examined cell lines and displayed elevated levels under hypoxia. The results of cells treated with $CoCl_2$ are similar to hypoxia (Fig. 4). Quantitative real-time PCR confirmed these data by showing considerable increase of mRNA FL and AS under hypoxic conditions when compared to normoxia (Fig. 5).

3.5. Promotion on migration of HSC3 by hypoxia

Using a cell wound healing assay, it was shown that hypoxia significantly increased the migration of HSC3 cell in a time-dependent manner ($p<0.001$) (Fig. 6).

4. Conclusion

As far as we known, this is the first systematic attempt to evaluate the mRNA expression of FL and AS CAIX isoforms in human oral cancer in vitro levels. We have demonstrated for the first time that both FL and AS CAIX are elevated in hypoxic conditions than normoxia. Data from our in vitro experiments showed that hypoxia was capable of stimulating FL and AS CAIX mRNA expression in human oral cancer cells. This suggests that one of the pathogenic mechanisms of OSCC in vivo may be the synthesis of CAIX by resident cells in response to hypoxia. In addition, CAIX induced cell migration by hypoxia. Therefore, studying the signal transduction pathway involved in CAIX expression may prove versatile. However, more detailed studies should be undertaken to clarify that hypoxia can regulate CAIX in vitro and in vivo. Further studies will be important to address the contribution of hypoxia in the OSCC.

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Table 1. The sequences of primers used were as follows:

Primer designation	Position	Sequence (5'- 3')
h1S	412-433	GAACCCCAGAATAATGCCCACA
h6A	924-945	TCGCTTGGAAGAAATCGCTGAG
h7S	980-1001	TATCTGCACTCCTGCCCTCTG
h8A	1133-1155	CACAGGGTGTCAGAGAGGGTGT
h10/7A	1291-1279 / 1106-1095	CTAGGATGTCAC / CTGCTTAGCACTC

Figure Legends

Fig. 1. Expression of CA IX mRNA and protein in human oral cancer cell lines. (A) The mRNA of cells were prepared and analyzed by RT-PCR using h1S-h6A primers. GAPDH gene was performed in order to monitor equal RNA loading. (B) The total cell lysates were subjected to Western blot to analyze the expressions of CA IX. β-actin is an internal control.

Fig. 2. Expression of CA IX mRNA and protein in human oral cancer cell lines exposed to normoxic and hypoxic conditions. Six cell lines were treated with normoxia (N), hypoxia (H) or $CoCl_2$ for 24 hours. The CAIX mRNA (A) and protein (B) levels of cells were analyzed using RT-PCR and Western blot assay, respectively. The levels of CA IX mRNA and protein under hypoxic conditions are significantly increased than normoxic. GAPDH gene and β-actin protein are internal controls.

Fig. 3. Expression of FL-CA IX and AS-CA IX mRNAs in human oral cancer cell lines. RT-PCR analysis of human AS-CA IX using the primers designed for individual amplification of the splicing variants, namely h7S-h8A for FL and h7S-h10/7A for AS. GAPDH is used as a standard.

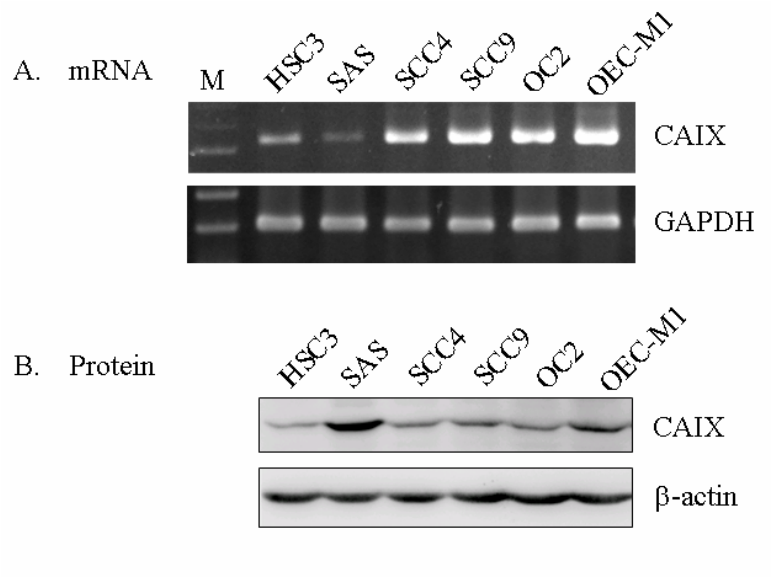
Fig. 4. Expression of FL-CA IX and AS-CA IX mRNAs in human oral cancer cell lines exposed to normoxic and hypoxic conditions. The mRNAs of six cell lines were extracted under normoxia (N), hypoxia (H) or $CoCl_2$ for 24 hours. The results indicate

that both FL and AS expression are great depend on hypoxia. GAPDH is used as an internal control.

Fig. 5. Expression of FL-CA IX, AS-CA IX and CA IX mRNA in human oral cancer cell lines exposed to normoxic and hypoxic conditions. The data of RT-PCR were confirmed by quantitative real-time PCR. The results also showed considerable increase of FL, AS and CA IX under hypoxic conditions when compared to normoxia. The amount of each type of PCR product was normalised against GAPDH.

Fig. 6. Effect of hypoxia on the migration of HSC3 cells. HSC3 cell monolayers were scraped by a sterile micropipette tip, and cells were exposed to normoxia and hypoxia for 0, 12 and 24 h. The number of cells in the denuded zone was quantified at the indicated times (0, 12 and 24 h) using an inverted microscope.

Figure 1.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M
HSC3
SAS
SCC4
SCC9
OC2
OEC-M1
CAIX
GAPDH
B. Protein
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SAS
SCC4
SCC9
OC2
OEC-M1
CAIX
β-actin

Figure 2.

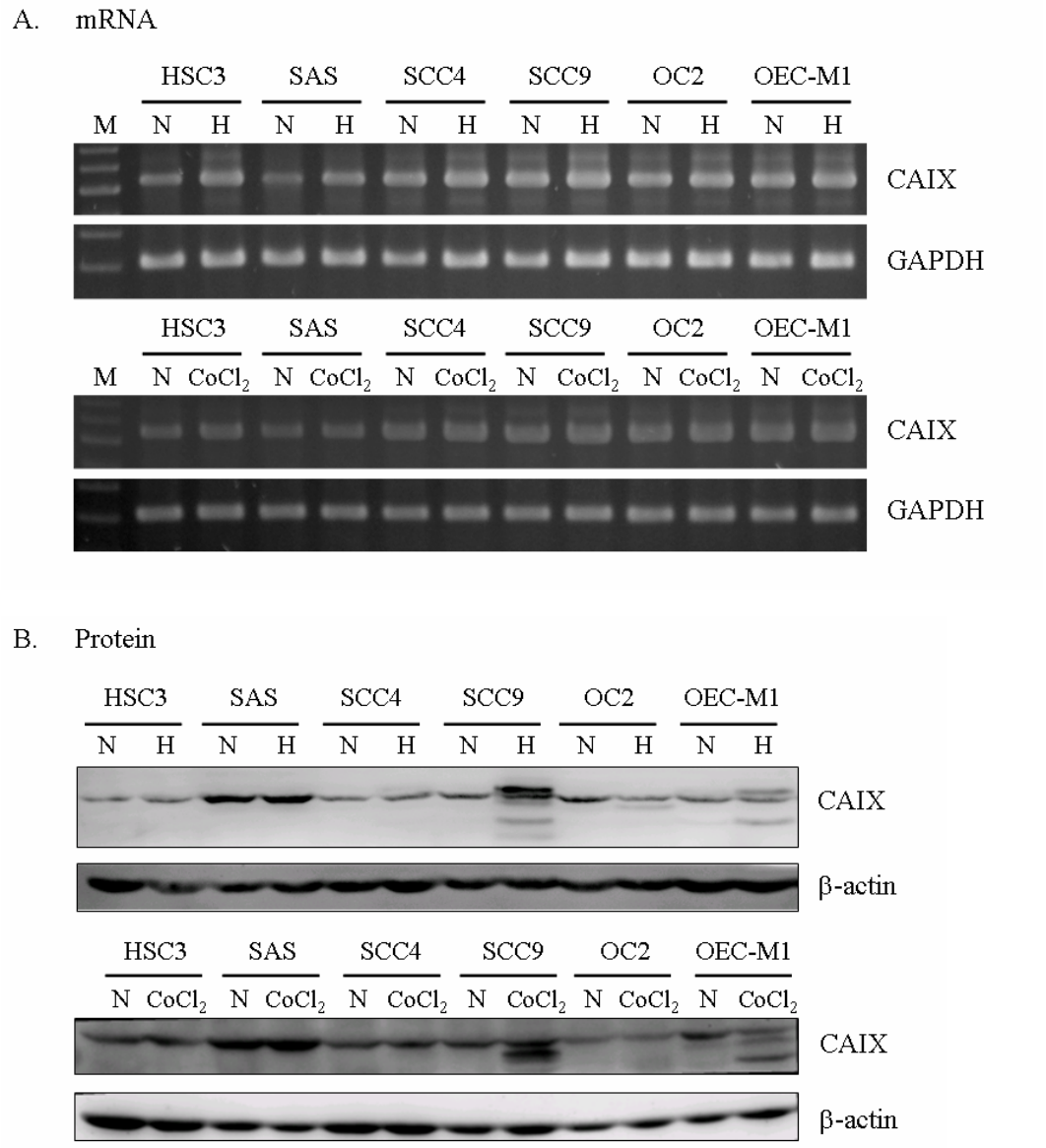
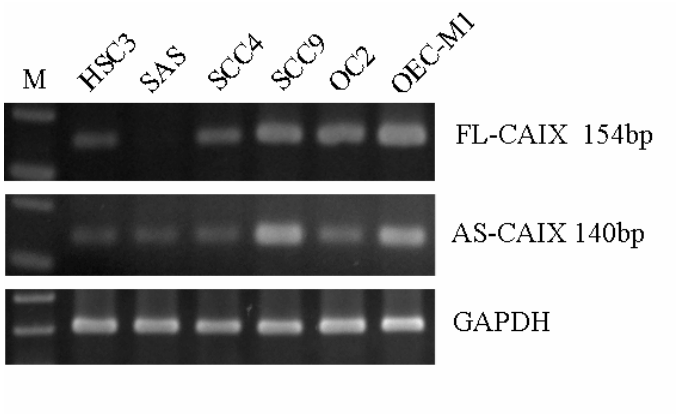

A. mRNA
HSC3 SAS SCC4 SCC9 OC2 OEC-M1
M N H N H N H N H N H N H
CAIX
GAPDH
HSC3 SAS SCC4 SCC9 OC2 OEC-M1
M N CoCl2 N CoCl2 N CoCl2 N CoCl2 N CoCl2 N CoCl2
CAIX
GAPDH
B. Protein
HSC3 SAS SCC4 SCC9 OC2 OEC-M1
N H N H N H N H N H N H
CAIX
β-actin
HSC3 SAS SCC4 SCC9 OC2 OEC-M1
N CoCl2 N CoCl2 N CoCl2 N CoCl2 N CoCl2 N CoCl2
CAIX
β-actin

Figure 3.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SAS
SCC4
SCC9
OC2
OEC-M1
FL-CAIX 154bp
AS-CAIX 140bp
GAPDH

Figure 4.

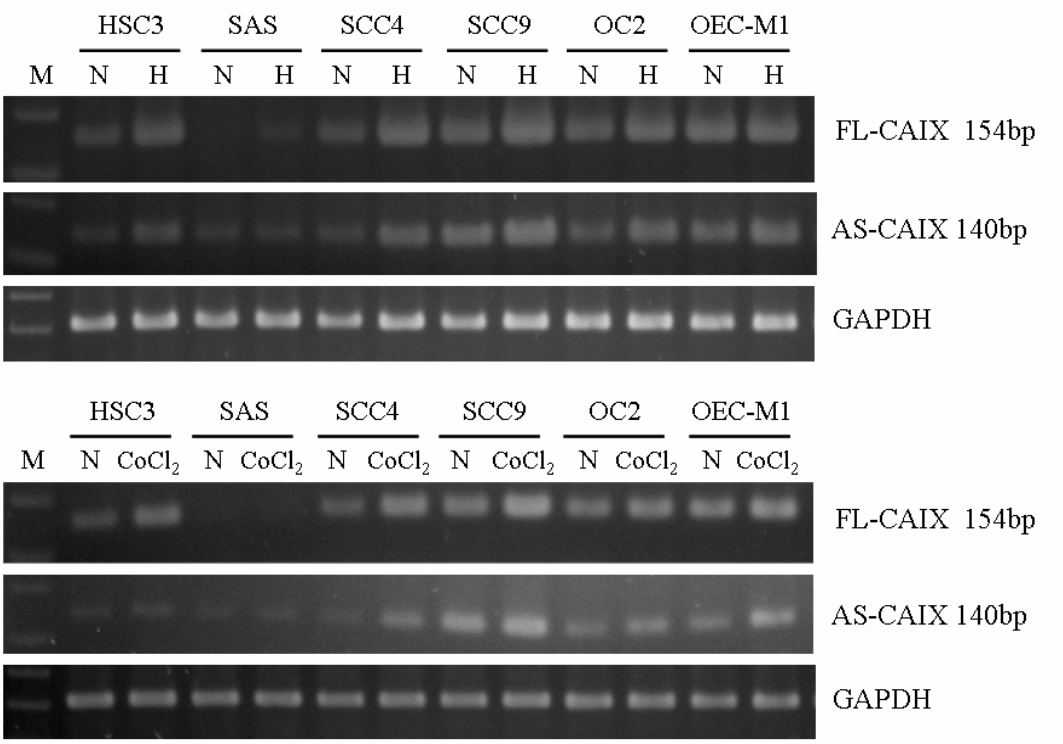
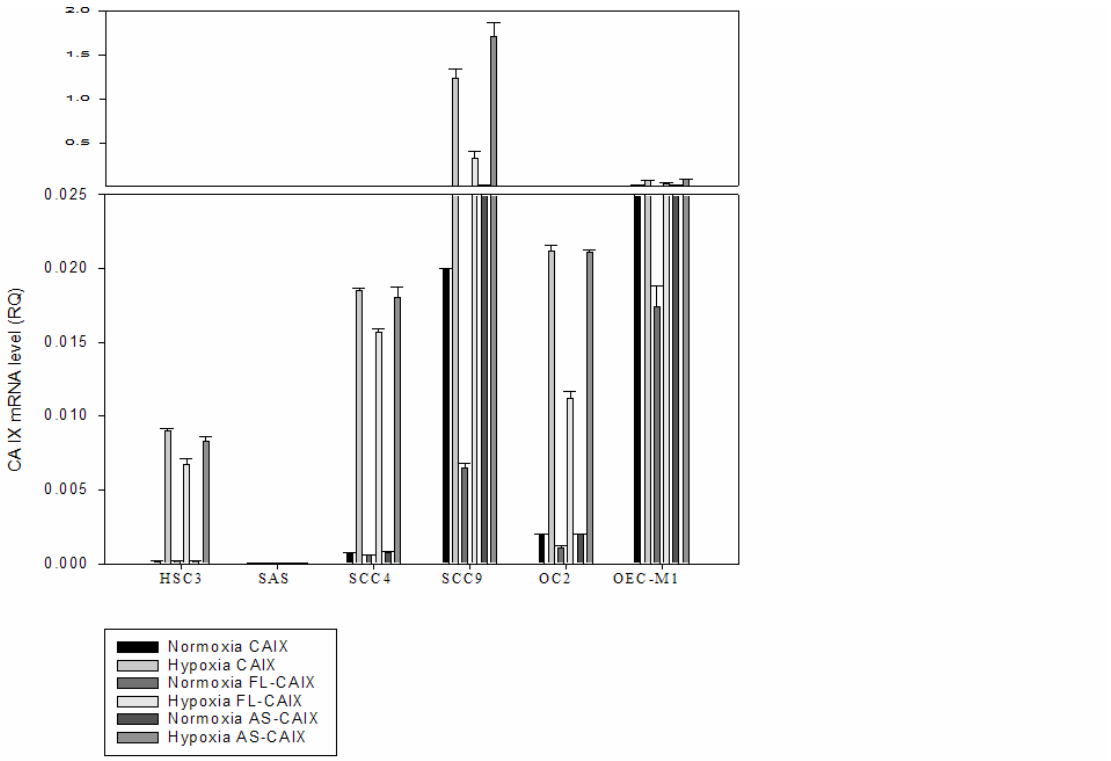

HSC3
SAS
SCC4
SCC9
OC2
OEC-M1
M
N
H
FL-CAIX 154bp
AS-CAIX 140bp
GAPDH
HSC3
SAS
SCC4
SCC9
OC2
OEC-M1
M
N
CoCl2
FL-CAIX 154bp
AS-CAIX 140bp
GAPDH

Figure 5.

A.


CA IX mRNA level (RQ)
2.0
1.5
1.0
0.5
0.025
0.020
0.015
0.010
0.005
0.000
HSC3
SAS
SCC4
SCC9
OC2
OEC-M1
Normoxia CAIX
Hypoxia CAIX
Normoxia FL-CAIX
Hypoxia FL-CAIX
Normoxia AS-CAIX
Hypoxia AS-CAIX

B.

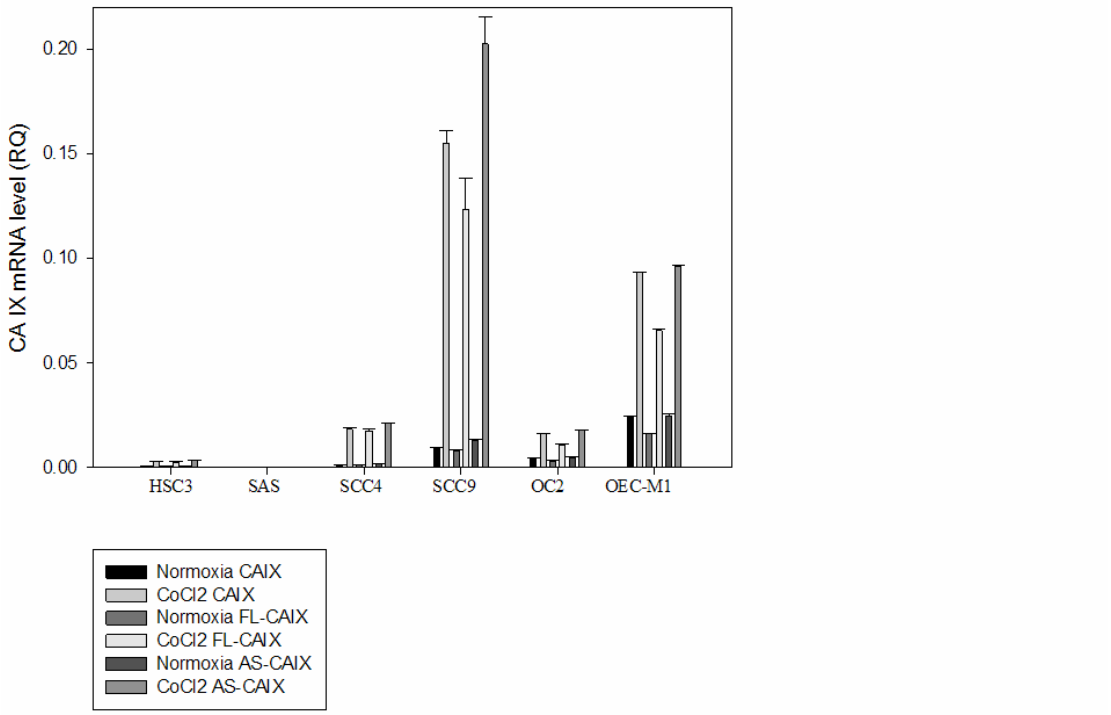
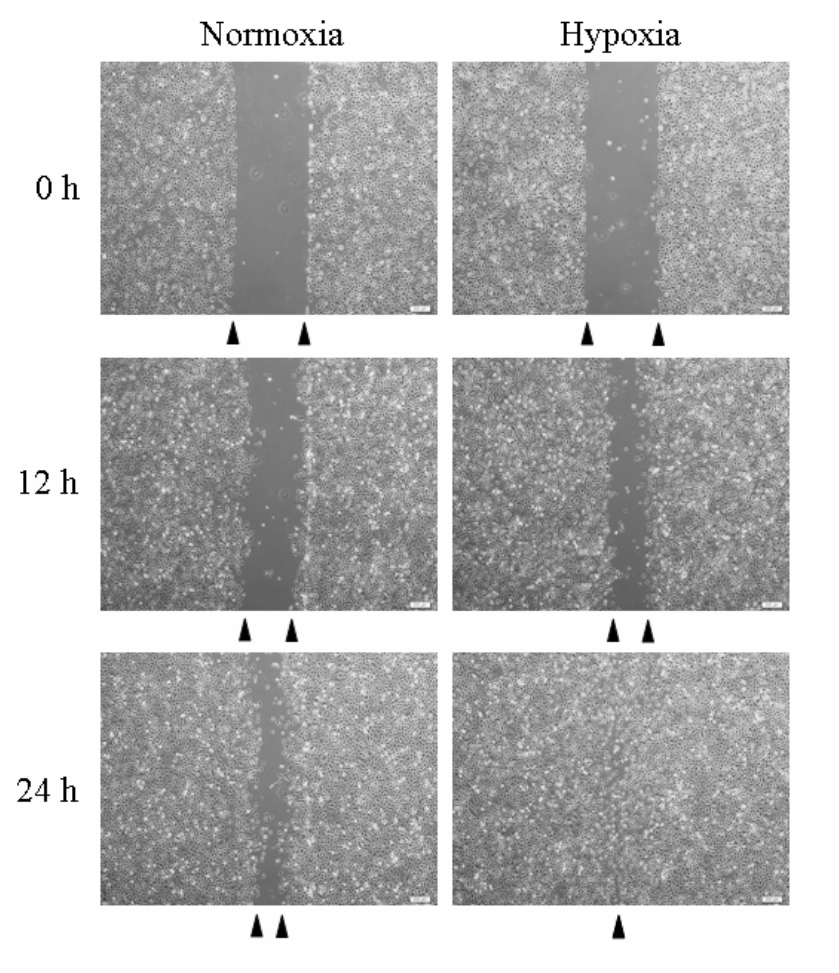

CA IX mRNA level (RQ)
0.20
0.15
0.10
0.05
0.00
HSC3
SAS
SCC4
SCC9
OC2
OEC-M1
Normoxia CAIX
CoCl2 CAIX
Normoxia FL-CAIX
CoCl2 FL-CAIX
Normoxia AS-CAIX
CoCl2 AS-CAIX

Figure 6.



行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

99 年 10 月 28 日

附件三

報告人姓名	楊順發	服務機構及職稱	中山醫學大學 醫學研究所 副教授
時間 會議 地點	99 年 7 月 13 日至 7 月 18 日 西班牙巴塞隆納	本會核定 補助文號	
會議名稱	（中文）第 88 屆國際聯合牙醫研究會議 （英文）88 th General Session & Exhibition of the International Association for Dental Research		
發表論文題目	（中文）MMP-14 基因多型性與口腔癌的相關性探討 （英文）MMP-14 polymorphism is Risk Factor for oral cancer susceptibility		

報告內容應包括下列各項：

一、參加會議經過

於 7 月 14 日抵達西班牙巴塞隆納後即前往會議地點（巴塞隆納國際會議中心）報到及領取大會議程及摘要手冊，將準備好的論文海報張貼於指定位置。並於期間前往會場聆聽多場特別演講，並在下午於展出海報處說明研究成果。展示期間，多位學者表示對本研究的高度興趣，並有多方討論。

二、與會心得

本人的研究主題主要是在 MMP-14 基因多型性與口腔癌的相關性探討，而本次大會的主題除了涵蓋人類口腔黏膜下纖維化的基礎研究之外，還加入臨床治療及個案探討，因此藉由此次會議讓我有機會接觸到更實際的臨床領域，獲得不少新觀念及之前未曾有過的一些想法。會中聆聽許多大師級的演講，受益良多。與其他相關研究人員的諸多討論，也獲得很多寶貴的意見及肯定。此類與國外研究人員的溝通及聯繫是很重要的，讓我有機會與國外實驗室有初步之合作構想，並已有初步之計畫，希望能藉此有國際合作的機會。

三、建議

國內應多加舉辦如此大型會議、增加補助出國額度、或盡量補助博士班學生出國開會或短期研究之經費，讓年輕研究學者有機會與大師級學者學習。

四、攜回資料名稱及內容

會議議程手冊
會議摘要手冊

無衍生研發成果推廣資料

96 年度專題研究計畫研究成果彙整表

計畫主持人：楊順發	計畫編號：96-2628-B-040-021-MY3
計畫名稱：碳酸酐異構酶 IX, XII 在口腔黏膜下纖維化及口腔癌的表現	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	2	2	100%	人次	
		博士生	2	2	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	3	3	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 ■未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

碳酸酐酵素 IX (Carbonic anhydrase IX, CAIX)是穿膜糖蛋白(transmembrane glycoprotein)屬於鋅酵素 (zinc metalloenzyme)家族的一員，可逆的催化 CO2 及 H2O 轉換成 HCO3-及 H+，幫助細胞調節離子運輸及維持酸鹼平衡。CAIX 主要表現在癌細胞上，在缺氧的情況下可使細胞內過多的氫離子排出及酸化細胞外環境，更有利於癌細胞生長。本計畫主要針對口腔癌其 CAIX 的 DNA polymorphism,mRNA expression 及 splicing 及 protein expression 的表現做探討。目前已有初步成果並正在投稿中。初步結果發現 CAIX 的 polymorphism 與口腔癌的罹癌機率有關。但其 RNA splicing 並不影響其癌細胞生長及轉移。