

# 美和科技大學

## 106 年度教師專題研究計畫 結案報告

總計畫名稱：以體外(in vitro)試驗探討檳榔與綠茶在食道腫瘤的發生、轉移及預後之影響

**Effect of areca nut and green tea on esophageal cancer carcinogenesis and prognosis by *in vitro* study**

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## 中文摘要

食道癌占國人男性癌症死亡率的第六名，其五年存活率少於 40%。雖然我們先前臨床流行病學研究已經發現除了抽菸與喝酒外，嚼食檳榔也是國人罹患食道癌之重要危險因子，相較於只有抽菸習慣者，有抽菸及嚼食檳榔兩種習慣者罹患食道癌的危險性增加 1.8 倍；抽菸、喝酒及嚼食檳榔的習慣中只要有兩個習慣者，其罹患食道癌的危險性為 3.8 到 20.4 倍，如果同時抽煙、喝酒及嚼食檳榔，則罹患食道癌的危險性增加到 41.2 倍。在台灣地區，抽菸族群中有 33.6% 具嚼食檳榔的習慣，喝酒族群中有 35% 具嚼食檳榔的習慣，因此嚼食檳榔對於台灣人所罹患的食道癌相對的重要。另外，我們流行病學研究亦證實，不發酵茶對於食道癌的發生具有保護作用，綠茶為國人常喝的不發酵茶，我們將利用台灣人的食道癌細胞株 (81T/VGH) 及西方人食道癌細胞株 (OE21) 添加檳榔鹼及綠茶/ EGCG，探討檳榔鹼及綠茶/ EGCG 對食道癌細胞株的生長及侵犯性的影響。

## 英文摘要

Esophageal cancer is the 6th leading cause of cancer death in Taiwanese males. Once diagnosed with ESCC, the prognosis is very poor with 5-year survival rate below 40%. Our previous studies have reported, besides cigarette smoking and alcohol drinking, areca nut chewing was one of the major risk factors for esophageal squamous cell carcinoma (ESCC) in Taiwanese population. Combined exposure to any two of three substances brought up the risk from 3.8-fold to 20.4-fold and, to all 3 substances, to 41.2-fold. Areca nut use occurs in 33.6% of the smoking population and in 35% of those who consume alcohol. Unlike other Southeast Asian countries, betel nut is not chewed with tobacco in Taiwan. Since there is no concrete evidence to verify the epidemiological findings, we present an animal model to investigate the effects of betel quid chewing on the development of esophageal cancer. Meanwhile, we also found significant inverse relationship between the frequency of tea consumption and esophageal cancer. Green tea is the most popular unfermented tea in Taiwan. Combining the results of *in vitro* studies should provide a better chance for developing therapeutic drugs for esophageal cancer.

## 前言

### **Epidemiological data**

Of all cancer types worldwide, esophageal cancer has the sixth highest mortality rate (5.0/100,000).[1] In Taiwan, 2012 data indicate that, of all cancer types, esophageal cancer had the ninth highest mortality rate in the overall population (5.2/100,000) and the fifth highest mortality rate in males (10.1/100,000) (DOH/ROC, 2014). Once diagnosed, the prognosis of esophageal cancer is very poor. The overall 5-year survival rate is < 40%, which indicates the importance of preventing the occurrence of esophageal cancer.[2-4] Since 1996, our research team has collaboratively elucidated the risks of esophageal squamous cell carcinoma (ESCC) in Taiwan because ESCC is the predominant histological type of esophageal cancer.[5, 6] Our epidemiological findings showed that esophageal cancer is 4.4 times more common in areca nut chewers than in non-chewers.[7] Subjects who smoke cigarettes and chew betel quid (BQ) have a 1.8-fold higher risk of developing esophageal cancer compared to subjects who only smoke cigarettes. The study also showed that subjects who drink alcohol and chew BQ have a 12.0-fold higher risk of developing cancer compared to subjects who only drink alcohol.[6] Our further studies revealed that patients who used areca nut simultaneously with another potentially addictive substance (tobacco or alcohol) developed ESCC at an age 7-8 years younger than those who used only areca nut, which suggests that areca nut may have an enhancing effect on carcinogenesis.[8]

### **Role of BQ chewing in esophageal cancer**

Chewing areca nut is quite common in several areas of Southeast Asia, in some South Pacific islands, and in Taiwan. Areca nut chewing is strongly associated with both esophageal cancer[5, 6] and oral cancer.[9] In Taiwan, areca nut is chewed in the form of the raw betel fruit with Piper betel inflorescence or folded into a betel leaf. Areca nut is the main psychoactive substance and an important environmental risk factor for development of cancer. The areca nut without tobacco has been recognized as group I carcinogen in 2004 by the World Health Organization classified.[10] Given the increasing incidence and mortality rate of esophageal cancer, identifying its risk factors and molecular mechanisms is an essential task.

### **Toxicity of arecoline**

Arecoline, the major alkaloid of areca nut, has *in vivo* and *in vitro* cytotoxic and genotoxic effects in mammalian cells and even contributes to carcinogenicity.[11-15] A 1983 study found that arecoline produces carcinogenic N-nitrosamines. *In vitro* experiments reported here have shown that N-nitrosation of arecoline leads to N-nitrosoguvacoline (NG), 3-(methylnitrosamino) propionitrile

(MNPN) and 3-(methylnitrosoamino) propionaldehyde.[16] The documented *in vitro* cytotoxic effects of arecoline can be divided into three stages: first, inhibition of cellular growth and attachment, synthesis of collagen synthesis, and mitochondrial cytochromeoxidase activity; second, depletion of cellular glutathione; third, arrest of the cell cycle in G2/M phase.[11, 14] Furthermore, *in vivo* and *in vitro* studies of mouse tissues and cells indicate that arecoline has potential tumorigenic effects. Genotoxic effects include induction of chromosome aberrations, sister chromatid exchange, micronuclei formation, and DNA strand breaks.[12, 13, 15] Metabolites derived from N-nitrosation of arecoline in saliva during betel nut chewing are also potentially tumorigenic.[17] The potential cytotoxic and genotoxic effects of arecoline exposed in betel nut chewers were well recognized. In our animal study, an F344 rat model was used to investigate the role of arecoline on esophageal tumorigenesis. The study data indicate that arecoline promotes esophageal tumorigenesis induced by carcinogen N-nitrosomethylbenzylamine (NMBA) in F344 rats.

### **Effects of arecoline and BQ on tumorigenesis and metastasis**

A previous *in vitro* study used a selection model to analyze cultured CE81T/VGH cells (parent cells) after repeated exposure to BQ extract (BQE) and arecoline (selected cells). Long-term stimulation apparently accelerated migration in the surviving cells. Compared to parent cells, the arecoline- and BQE-selected CE81T/VGH cells had significantly higher motility ( $p=0.009$  for both).[18] In oral squamous cell carcinoma (OSCC) associated with areca quid chewing, overexpression of *Drosophila* embryonic SNAIL1 (Snail) is reportedly associated with tumor differentiation and lymph node metastasis. Arecoline-upregulated Snail expression may be mediated by reactive oxygen species (ROS) generation. Arecoline-induced Snail expression is also downregulated by NAC, curcumin, and epigallocatechin 3-gallate (EGCG).[19]

### **Role of green tea in esophageal cancer**

Various epidemiological studies substantiate the correlation between green tea consumption and cancer prevention. A recent meta-analysis of twenty-four case-control and cohort studies comprising 7376 esophageal cancer cases found that consuming green tea, but not black tea, has protective effects against esophageal cancer.[20] The findings of our epidemiological study further show a significant inverse relationship between the frequency of tea consumption and esophageal SCC risk ( $p$  for trend = 0.005). Regular intake of unfermented tea is associated with a 0.5-fold lower risk of esophageal cancer.[21] The protective effects of green tea, which is the most popular unfermented tea in Taiwan, are believed to result from its polyphenols.

Many laboratories have demonstrated that green tea and its polyphenol preparations inhibit tumorigenesis.[22] Green tea is rich in polyphenol flavonoids, including catechins. The most abundant catechin in green tea is EGCG,[23] which is often classified as an antioxidant associated with cancer prevention. A review of the effects of green tea extract and EGCG on the signaling network in squamous cell carcinoma found that the major signaling pathways are epidermal growth factor receptor and the Notch pathway, which is affected by cell cycle-related networks.[24] Earlier animal models of carcinogenesis have shown that green tea fraction and EGCG inhibit tumorigenesis. Rat studies also show that EGCG significantly inhibits NMBA-induced esophageal cancer, and its inhibitory effects may partly target cyclin D1 expression, COX-2 expression, and PGE2 production.[25] In another rat study, the experimental group was given green tea fractions in drinking water for two weeks before administration of NMBA. The control group, which was treated with NMBA only, had a 100% incidence of multiple esophageal tumors ( $3.3 \pm 0.4$  tumors per rat). In contrast, the rats given green tea fraction before NMBA had a lower incidence of multiple esophageal tumors. The incidence of multiple tumors was also significantly lower in rats given green tea fraction than in rats given NMBA only. This research indicates that treatment with theaflavins and EGCG significantly reduces esophageal tumor formation rates.[26] Various mechanisms of the inhibiting effects of green tea on carcinogenesis have been proposed. However, the protective effects against development of cancer, especially arecoline-induced esophageal tumors, are unclear.

## 研究方法、步驟

### < **Research materials and methods** >

*In vitro* studies we have performed to analyze the effects of arecoline and green tea/epigallocatechin-3-gallate (EGCG) in esophageal cancer cell lines and soft agar colony-formation assay, MTX assay, wound-healing test, and *in vitro* migration and invasion assays. Molecular mechanism were include pAKT and pAKT protein expression.

To assay tumorigenic potential, *in vivo* studies will characterize the growth of cancer cells and tumorigenic potential in the subcutaneous space of immunocompromised mice. After injecting CE81T2-4 cells and OE21-1 cells ( $1 \times 10^6$  cells in 0.2 ml Hank buffer) into subcutaneous tissues in the dorsal region in six T-cell-deficient BALB/c Foxlnn mice, tumor development was assessed by weekly measurements of the tumor mass for 8 weeks. Metastasis was assessed by injecting cell lines into the tail veins of the T-cell-deficient mice.

### **Cell lines and culture conditions**

A Taiwanese esophageal cancer cell line CE81T/VGH and a Caucasian cell line OE21 will be used for this study. An esophageal cancer cell line, CE81T/VGH (BCRC 60166), which was obtained from a 57-year-old Taiwanese male, will be provided by the Food Industry Research and Development Institute (Hsinchu City, Taiwan; <http://www.firdi.org.tw/index.htm>). Cells will be grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco BRL) and kept at 37°C in a 5% CO<sub>2</sub>/95% air atmosphere. The other ESCC cell line, OE21 (ECACC 96062201), which was obtained from a 74-year-old Caucasian male, will be purchased from the European Collection of Animal Cell Cultures (ECACC) (Salisbury, UK). Cells will be cultured in RPMI 1640 supplemented with 10% FBS (Gibco BRL), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B and 292 µg/ml L-glutamine (Invitrogen) and kept at 37°C in a 5% CO<sub>2</sub>/95% O<sub>2</sub> air atmosphere. Additional invasive esophageal cancer cell lines, including Taiwanese ESCC cell line CE81T2-1 and Caucasian ESCC cell line OE21-2, will be used for *in vivo* animal study. Transwell invasion chambers were used to select particularly invasive sublines.[27, 28]. The invasive characteristics of CE81T/VGH cell subpopulation (CE81T2-4) were confirmed both by *in vitro* and/or *in vivo* assays.[27] The OE21-1 subline was selected from OE21 cells.

## **Western blot**

Cells were lysed in 100  $\mu$ L RIPA buffer for 30 min on ice. Then, cell lysates were centrifuged at 13,000 g at 4°C, and the protein concentrations determined with Bradford reagent using bovine serum albumin as standards. Clarified protein lysates (20 $\mu$ g) were electrophoretically resolved on denaturing SDS PAGE gel (10%), a total volume of 20  $\mu$ g of protein was loaded per lane. Then, transferred to PVDF membranes (Millipore). The blots were blocked with TBS-T (20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at room temperature for 30 minutes, and TBS-T containing 5% bovine serum albumin for 90 minutes. After three washes with TBS-T for 10 minutes, the membranes were incubated with various antibodies: Akt, Phospho-Akt (Thr308), Phospho-Akt (Ser473), p44/42 MAPK (Erk1/2), Phospho-p44/42 MAPK (Erk1/2)(Cell Signaling),  $\beta$ -actin (Sigma). The following dilutions were used: Akt, Phospho-Akt (Thr308), Phospho-Akt (Ser473), p44/42 MAPK (Erk1/2), Phospho-p44/42 MAPK (Erk1/2) 1:1000, and  $\beta$ -actin 1:10000. After primary antibody incubation, the membranes were incubated with HRP-labeled secondary antibody at a concentration of 1:10,000. After a series of washing steps, protein expression was detected by chemiluminescence using an ECL detection kit (Pierce™ ECL Western Blotting Substrate, Thermo) and then exposed with Fuji X-ray film.



## 研究結果與討論

The patients who used at least one substance, alcohol, tobacco and/or areca nut were referred to the substance users. In our study, the substance users who did not have green tea consumption habit were diagnosed with ESCC younger than the patients who had tea consumption habit but no any indicated substance use ( $58.20\pm 11.42$  vs  $71.14\pm 6.94$ ;  $p=0.01$ ). Similar result was acquired after adjusting for educational levels, study hospitals and clinical stages ( $p=0.003$ ). In addition, the substance users who did not have tea consumption habits suffered from ESCC 1.9-year younger than the substance users who have tea consumption habits after adjusting for educational levels, study hospitals and clinical stages ( $58.20\pm 11.42$  vs  $60.11\pm 10.9$ ;  $p=0.04$ ). These data suggested that the ingredients of green tea may have protective function on the substance use-associated ESCC.

**Table 1** Association between tea, areca, tobacco or alcohol used and diagnosed age of esophageal squamous cell carcinoma.

Tea	Areca	Tobacco	Alcohol	n (649)	Mean±SD	Median	β (95% CI)
- (<3 time/ week)	Any one substance use			389	58.20±11.42	58	-
- (<3 time/ week)	-	-	-	51	60.69±11.69	60	2.66 (-0.59~5.91)
+ (≥3 time/ week)	-	-	-	7	71.14±6.94	75	12.20 (3.11~21.29)
+ (≥3 time/ week)	Any one substance use			202	60.11±10.90	60	1.90 (-0.10~3.90)

Data were analyzed by one-way ANOVA ( $p=0.0046$ ), and significance was assessed by LSD *post hoc* test.

Abbreviations: SD, standard deviation; CI, confidence interval.

<sup>a</sup> Adjusting for education levels, study hospital and clinical stage.

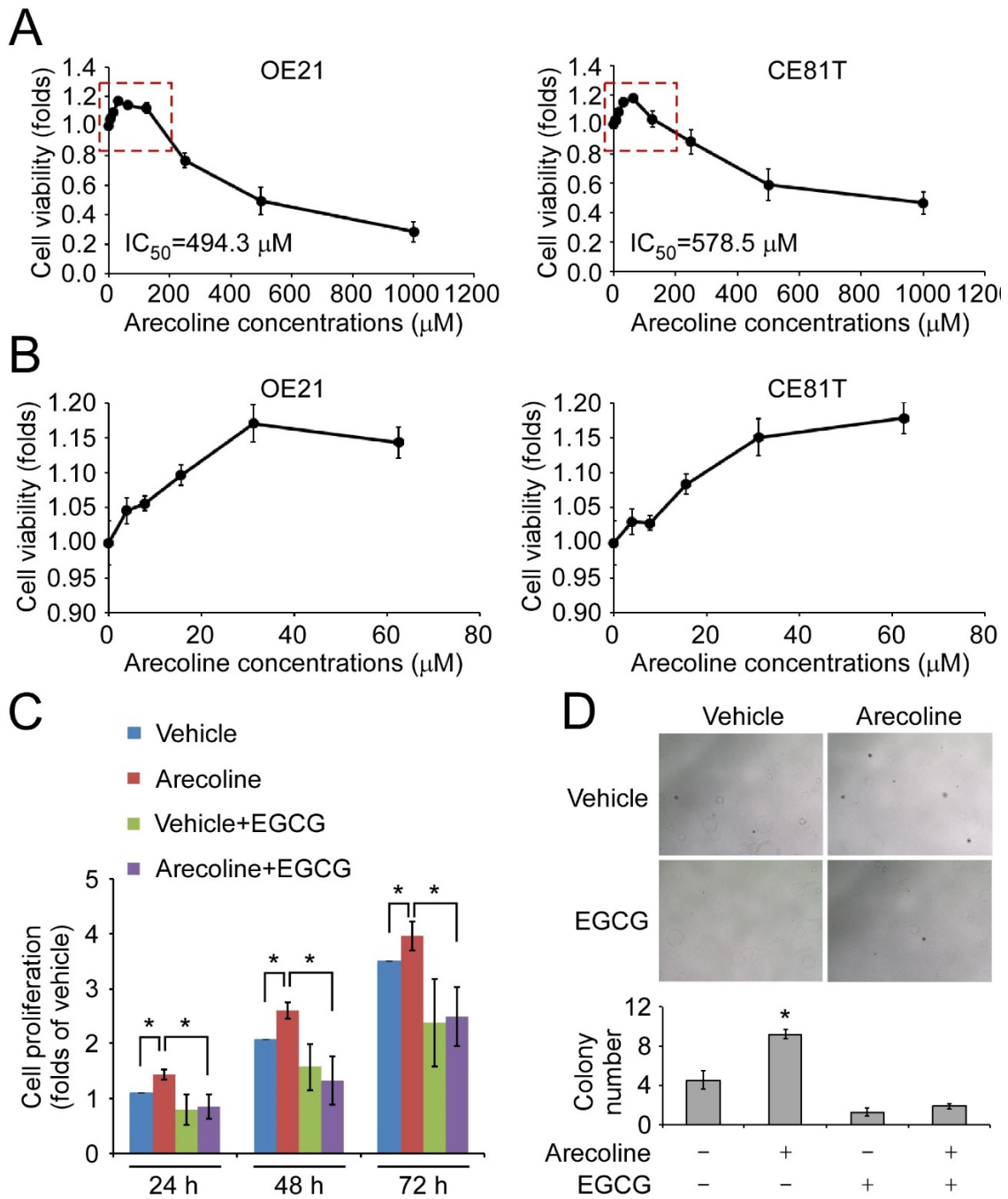
Animal study confirmed that arecoline facilitated the n-benzyl-n-methylnitrosamine-induced esophageal papilloma, suggesting the role of arecoline on promoting cell growth. The incidence of esophageal cancer was as follows: 14% (1/7) in the DMSO+NMBA group; 86% (6/7) in the DMSO+NMBA+arecoline group; 29% (2/7) in the DMSO+NMBA+arecoline+EGCG group; 14% (1/7) in the DMSO+NMBA+EGCG group; 29% (2/7) in the DMSO+NMBA+arecoline+green tea powder group; and 14% (1/7) in the DMSO+NMBA+green tea powder group. Notably, the incidence of papilloma in the DMSO+NMBA+arecoline group was significantly higher than those in the DMSO+NMBA+arecoline+EGCG group ( $p=0.031$ ) and the DMSO+NMBA+arecoline+green tea powder group ( $p=0.031$ ).

**Table 2. Incidence of esophageal papilloma**

No	Group	Number of esophageal papillomas (incidence, %)	<i>p</i> value*
1	DMSO (Control)	0, 0, 0, 0, 0, 0, 0 (0/7, 0%)	
2	DMSO + NMBA	0, 3, 0, 0, 0, 0, 0 (1/7, 14%)	0.299
3	DMSO + NMBA + Arecoline	3, 2, 1, 2, 1, 1, 0 (6/7, 86%)	0.001
4	DMSO + NMBA + Arecoline + EGCG	1, 1, 0, 0, 0, 0, 0 (2/7, 29%)	0.127
5	DMSO + NMBA + EGCG	0, 0, 0, 1, 0, 0, 0 (1/7, 14%)	0.299
6	DMSO + NMBA + Arecoline + Green tea powder	0, 0, 0, 1, 2, 0, 0 (2/7, 29%)	0.127
7	DMSO + NMBA + Green tea powder	0, 0, 1, 0, 0, 0, 0 (1/7, 14%)	0.299

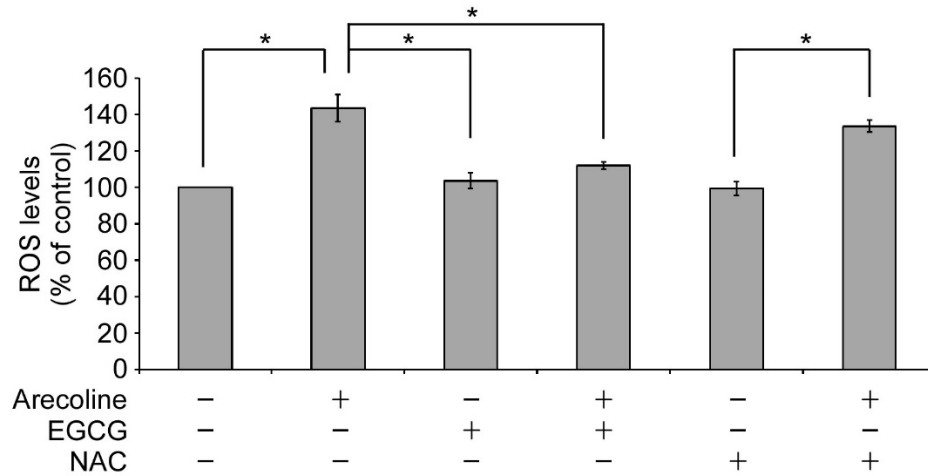
\*in comparison with Control group. Group 2 vs. Group 3: *p* value= 0.008; Group 3 vs. Group 4: *p* value= 0.031;

Group 3 vs. Group 6: *p* value= 0.031; Group 6 vs. Group 7: *p* value= 0.515

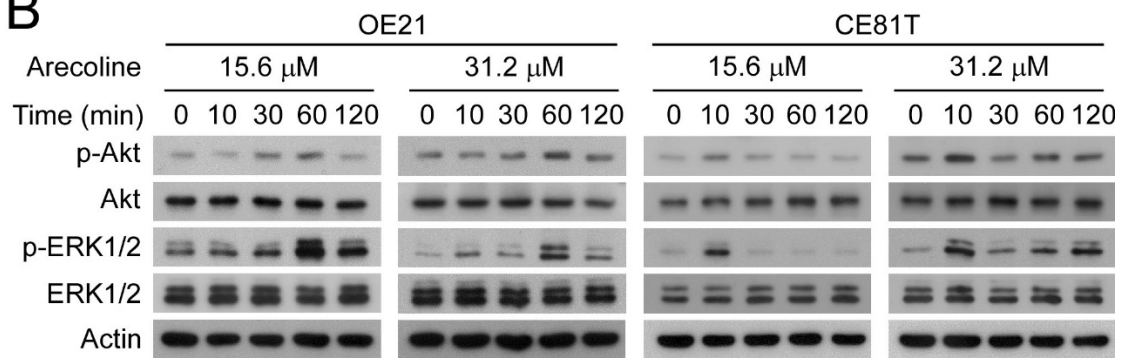


**Figure 1. EGCG constrains arecoline-induced proliferation of ESCC cell lines.**

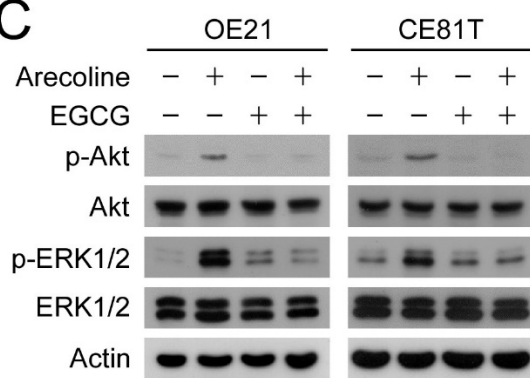
**A**



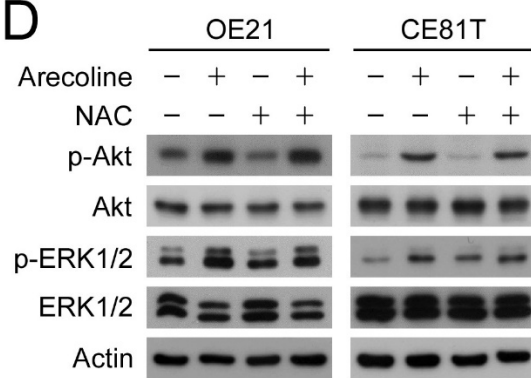
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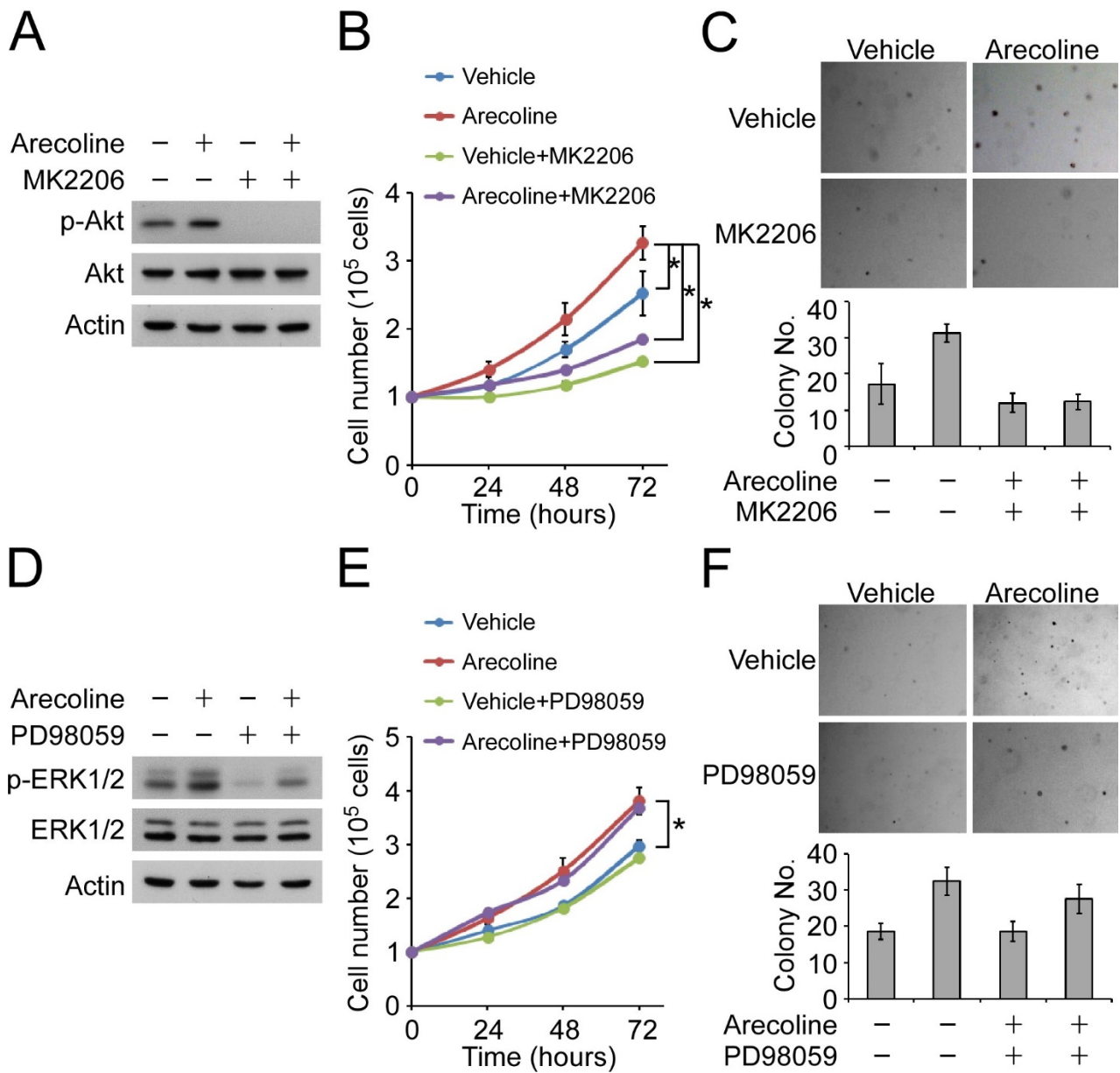
**C**



**D**



**Figure 2. EGCG inhibited arecoline-induced PI3K/Akt and ERK signaling pathways in a ROS-independent manner.**

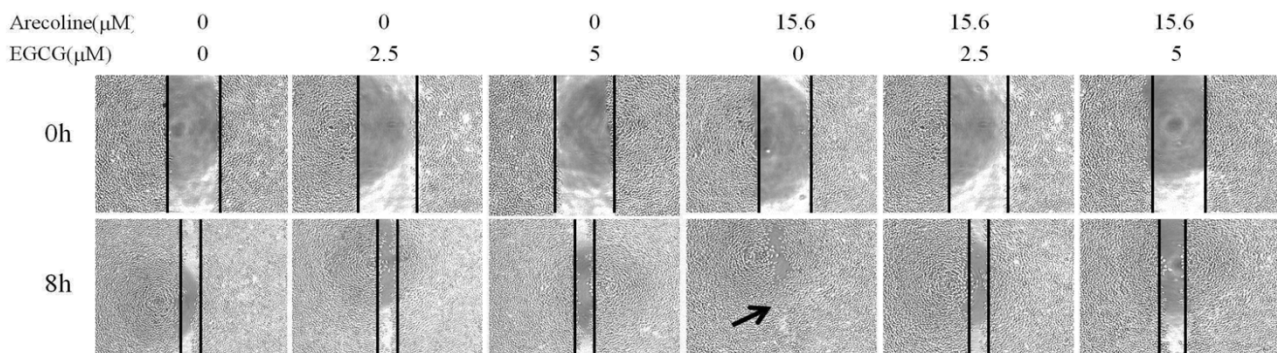


**Figure 3. Arecoline promoted cell proliferation through the activation of Akt signaling.**

## **Invasion and metastasis**

### **Wound-healing test to analyze OE21 motility**

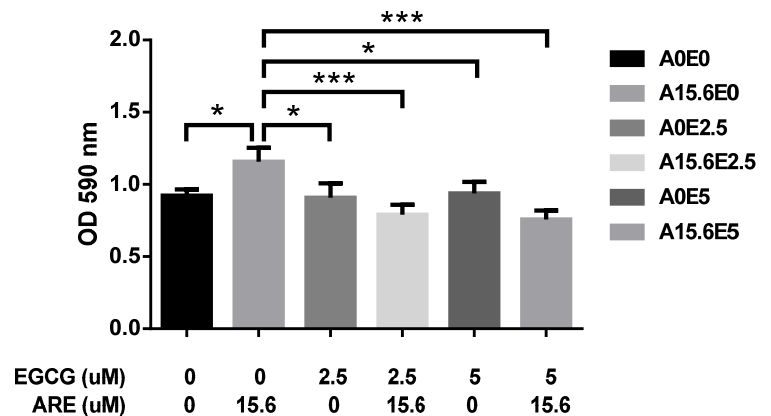
In this experiment, 15.6  $\mu\text{M}$  of arecoline and/or 2.5  $\mu\text{M}$ -5  $\mu\text{M}$  EGCG was added to OE21 cells in culture medium for 8 hours. An 8-hour culture was selected because wound healing was complete after the 24-hour migration assay. Distances between cells were measured at 0 and 8 hours after separation of cells in the wound healing assay. Migration distance was calculated by subtracting the distance measured at 8 hours from the distances measured at 0 hours. Figure 7 shows that the migration distance in the arecoline group ( $447 \pm 16.0$  microns) was significantly longer than those in the control group ( $365 \pm 9.6$  microns) and the arecoline+EGCG group ( $328 \pm 14.7$ ) (all  $p < 0.05$ ). This comparison was based on representative data from three independent experiments performed in quadruplicate.



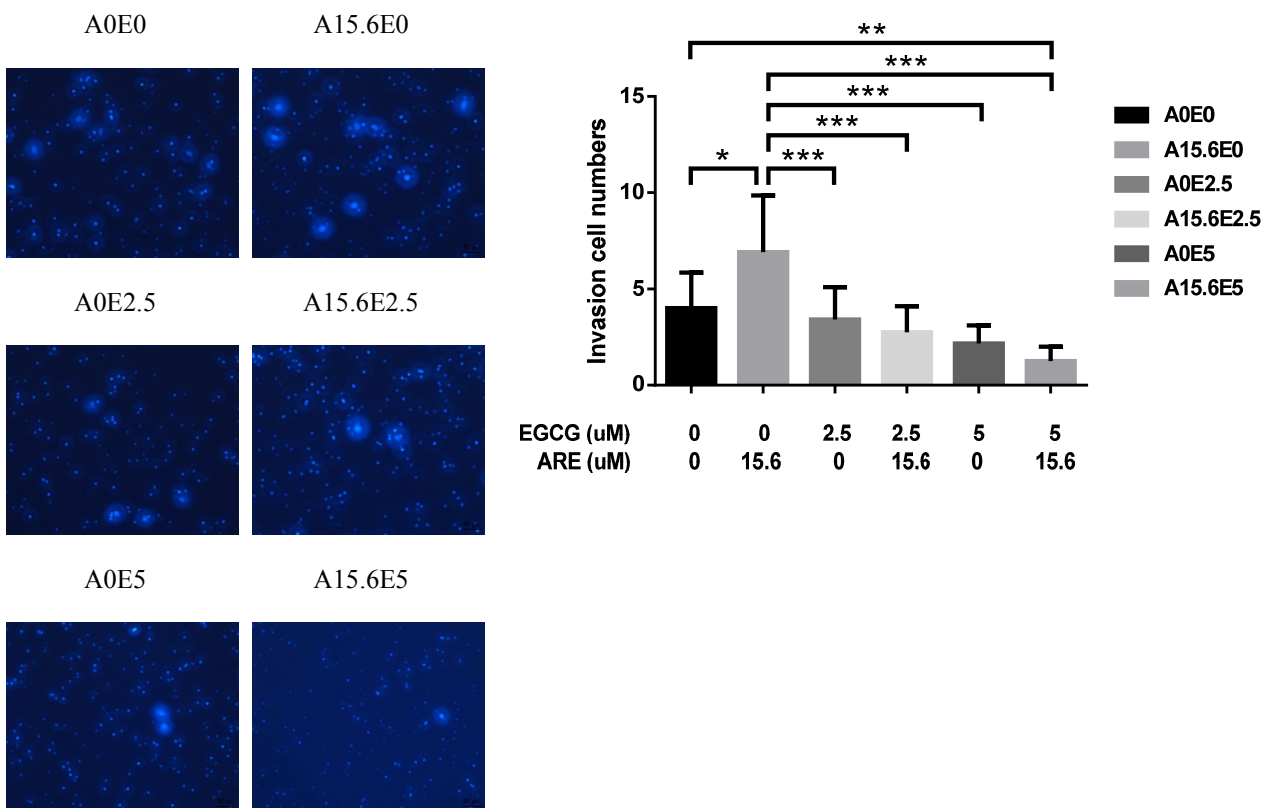
**Figure 4. Wound-healing assay of confluence of OE21 cell migration.** Data are representative of three independent experiments performed in quadruplicate (original magnification,  $\times 100$ ).

## Chamber migration and invasion assay to analyze OE21 motility

After short-treatment arecoline 15.6 (A)  $\mu\text{M}$  and EGCG 2.5 and 5  $\mu\text{M}$  for 24 hours, arecoline could significant increase OE 21 cell migration and invasion ability and co-treat with EGCG could inhibit the cell migration and invasion ability (Figure 5).



(B)



**Figure 5. *In vitro* effect of EGCG and arecoline on OE21 cell line.** (A) migration assay determined by crystal violet . After short-treatment arecoline 15.6  $\mu\text{M}$  and EGCG 2.5 and 5  $\mu\text{M}$  for 24 hours, arecoline could significant increase OE 21 cell migration and co-treat with EGCG could inhibit the cell migration.; (B) invasion assay determined by DAPI. After short-treatment arecoline 15.6  $\mu\text{M}$  and EGCG 2.5 and 5  $\mu\text{M}$  for 24 hours, arecoline could significant increase OE 21 cell invasion ability and co-treat with EGCG could significant inhibit cell invasion ability.

## 結論

We preliminary used a murine model of esophageal papilloma by chemical induction with *N*-nitrosomethylbenzylamine (NMBA) to investigate whether green tea/ EGCG or arecoline have any effect in preventing or promoting formation of esophageal papilloma. The results strongly suggested that arecoline might be able to promote the effect of NMBA-induced papilloma tumorigenesis while green tea/ EGCG might suppress arecoline-promoted esophageal tumors. One Taiwanese ESCC cell line (81T/VGH) and Human Caucasian esophageal squamous carcinoma cell line (OE21) will be used for our study material. Cancer cell carcinogenesis characterization was performed by soft agar colony formation assay and cell proliferation assay. Cell motility, migration and invasion were analyzed by wound healing test, chamber migration, or invasion assay.



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