行政院國家科學委員會專題研究計畫 成果報告

以誘導表現系統、核酸酶及基因敲除鼠模型研究 gelsolin
在人類口腔癌變所扮演的角色及其機轉。

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中文摘要

根據衛生署 2002 年統計資料顯示，口腔癌在台灣佔男性十大癌症之第五位，且是目前擴展速度最快的癌症之一。快速的周邊組織侵犯及遠隔轉移是口腔癌臨床治療失敗之主因，細胞運動可能在其中扮演重要角色。已知肌動蛋白調控蛋白包括 gelsolin 等表現在細胞運動扮演重要角色。然而，口腔癌發展與 Gelsolin 之間的關係尚未被其他團隊研究過。前一期我們的研究已知在人類口腔癌中，gelsolin 表現呈現雙相變化。本研究主要目的在探討 Gelsolin 與口腔癌癒合及發展之關係。免疫組織化學染色分析發現正常組織較癌前組織與癌組織 Gelsolin 表現更多。然而，當癌細胞由癌前發展為癌或是發生轉移，Gelsolin 表現均有統計上的增加。此外，侵犯性生長模式、較大的腫瘤以及較年輕的口腔癌患者，Gelsolin 表現量均較高。病人的預後方面，Gelsolin 表現量的增加與較差的預後相關尤其在轉移癌族群。本次研究利用 Ecdysone 表現系統調節 gelsolin 表現，當超表現 gelsolin 於 OEC-M1 口腔癌細胞株可導致細胞生長減緩，進一步研究則發現是經由去氧核糖核酸合成速度下降，而非細胞衰亡增加所致。然而，當細胞以去除血清之刺激誘導凋亡進行，超表現 gelsolin 的細胞株其細胞凋亡的數目明顯增加。為進一步了解 gelsolin 的功能性角色與臨床上觀察到的組織進犯之關聯性，實驗中利用基因調控之口腔癌細胞模型以 Transwell chamber assay 及曠時攝影來評估。Gelsolin 確能促進細胞侵犯 Matrigel 的能力，然而對於細胞移動則呈現微抑製或是無關聯。顯示在機動蛋白調控外 gelsolin 於促進細胞進犯上另一特殊尚未被發現之途徑。在 gelsolin 之 epigenetic 表現調節方面，實驗中以組織蛋白去乙醯化之抑制劑觀察組織蛋白乙醯化對 Gelsolin 基因表現的作用，發現 gelsolin 表現明顯上升並且能執行其下游之功能性影響。然而去甲基化劑則無影響。顯示口腔癌中 gelsolin 表現可能是經由組織蛋白乙醯化而非 DNA 甲基化所調控。
Oral squamous cell carcinoma (OSCC) is now ranked the fourth-leading cancer deaths in Taiwanese male population, owing to the prevalent betel quid chewing habits. Regional invasion to vital organs and distant metastasis were the most common cause of the clinical failure, in which cell motility may play a critical role. Gelsolin was known to regulate cell morphology maintenance, motility, cellular differentiation, and apoptosis. It was one of the most common down regulated protein in many types of human malignancies. However, the role of gelsolin in oral carcinogenesis has not been evaluated. Our previous study demonstrated that gelsolin expression was decreased in oral precancer and cancer lesions compared with normal oral mucosa. Nevertheless, a significant positive association between gelsolin expression and the cancer progression was observed. Clinicopathological evaluation revealed that gelsolin expression was correlated with the size of the tumor, the invasive growth pattern, and earlier onset of the disease. Significant lower five-year survival rate was associated with increasing gelsolin expression in the tumor cells especially among the metastatic disease. In this study, the molecular mechanism of gelsolin expression in oral cancer cell motility and invasion, as well as cell growth and apoptosis was investigated using OSCC cell lines. The ecdysone expression system was used to increase the gelsolin level in OECM-1 cells. Overexpressing gelsolin in OECM-1 cells suppressed cell growth rate via inhibiting DNA replication rate rather than enhancing the apoptosis. However, gelsolin promoted cell apoptosis in response to serum deprivation. In addition, gelsolin expression does not or only slightly decrease cell motility, but significantly enhance cellular invasion into Matrigel. Treatment of OSCC cells with trichostatin A, a histone deacetylase (HDAC) inhibitor, enhanced gelsolin expression and promoted cancer cell death. However, there was no effect on 5-azacytidine (a demethylation agent) treatment. The data suggested that an epigenetic regulatory mechanism of histone acetylation could be one mechanism for gelsolin expression modulation in oral cancer cells. In summary, gelsolin expression is one significant biomarker of oral cancer progression. It may participate in oral carcinogenesis through the modulation of multiple functions in cell differentiation and apoptosis as well as cellular motility and invasion.

Keywords: gelsolin, oral cancer, cell growth, apoptosis, inducible expression system
報告內容

I. 前言:

Oral cancer includes cancers of the lip, tongue, cheek, other mouth site, and the oropharynx. More than 90% of oral cancers are squamous cell in origin. According to the Department of Health (DOH) in Taiwan, oral cancer account for 4.73% of all cancers with an annual incidence of 4.45 per 100,000 for male in 2002 (行政院衛生署, 2003). It was ranked the forth leading cancer in incidence and the fifth in mortality among males. The high incidence of oral cancer in Taiwan was attributed to betel quid chewing habits according to previous epidemiology studies (陳建仁, 1996). The mean age of oral cancer onset in Taiwan is significantly lower than those reported in the Western countries without betel quid chewing habits, such as US and UK (Worrall, 2001), which have about 10 years delay of disease onset.

The staging of oral cancer was based on three major parameters: the size of the primary lesion, the involvement of regional organs or tissues and the lymph nodes, and the presence of distant metastasis. Stage at diagnosis is associated with the disease prognosis. In Taiwan, the three-year and five-year survival rates for the early-stage (stage I and II) patients were 72% and 60%, respectively. For the late-stage disease (stage III and IV), they were 61% and 30%, respectively. However, oral cancer disease stage alone failed to precisely predict the clinical outcome for patients. Molecular genetic and pathological factors have been widely investigated in previous studies for their prognostic value (郭彥彬, 2000). However, all current prognostic measures still need to be improved for a better presentation of underlying biological aggressiveness of the disease and the patient’s outcome in addition to major discrepancies between the clinical significance of those molecular factors among different series of study. The role of cell motility associated factors in oral carcinogenesis and their prognostic value, however, have not been evaluated before.
II. 研究目的

The specific aim of this study is to continue our previous work of oral cancer clinical study and to investigate the role of gelsolin expression in the cellular apoptosis and proliferation, as well as cellular motility and invasion in the oral cancer cell line models via genetic and epigenetic manipulation of the protein expression.

III. 文献探討

Gelsolin is a ubiquitous actin binding protein that regulates the architecture and dynamics of cells through capping, severing, and nucleating actin filaments. Loss of heterozygosity (LOH) has been reported for the region encompassing the gelsolin locus in human bladder cancer (Tanaka et al., 1995). The structure and amino acid sequence of gelsolin are both highly conserved between different species in eukaryotic cells, which imply its importance in the cellular functions. The biological role of gelsolin, as its name self-explained, was first recognized to regulate the transition between “gel” and “sol” status of the cytoplasm through precise spatial regulation of actin monomer (G-actin) polymerization and depolymerization, which transformed cellular morphology for their functional needs and enabled cellular motility. Beside the “classical function” of gelsolin in the actin cytoskeleton regulation, gelsolin was recently identified to play certain role in the apoptosis signal transduction. Gelsolin is a prominent substrate for caspase-3 (Kamada et al., 1998; Kothakota et al., 1997), the common effector caspase in both the death receptor and mitochondrial apoptotic pathways.

Onset of the transformed / tumorigenetic phenotype correlates with the altered expression of actin-associated proteins (Pawlak and Helfman, 2001) including gelsolin. In cancer, the enhanced movements of malignant tumor cells that invade, destroy the normal tissue architecture, and metastasis is a major contributor to cancer progression.
(Button et al., 1995). These cancer-associated processes are based on actin dynamics. Although gelsolin functions as a promoting factor for fibroblast motility, the opposite observation was found in B16-BL6 mouse melanoma cells (Fujita et al., 2001). Gelsolin expression has been demonstrated to promote cellular invasion into type-I collagen matrix and precultured embryo chicken heart fragments depending on ras-rac signaling in the MDCK cell line model (De Corte et al., 2002).

In clinical studies, gelsolin has been reported to be down regulated in many types of solid tumors such as breast, lung, gastric, bladder, colorectal, prostate cancer, etc (Asch et al., 1996; Dosaka-Akita et al., 1998; Lee et al., 1999; Mullauer et al., 1993; Shieh et al., 1999; Tanaka et al., 1995; Vandekerckhove et al., 1990). It is one of the most significant genetic defects in malignancies by SAGE analysis (Zhang et al., 1997). However, overexpression of gelsolin was found to be a negative prognostic predictor in non-small cell lung cancer, urothelial cancer, and breast carcinomas (Rao et al., 2002; Shieh et al., 1999; Thor et al., 2001).

The role of actin regulatory proteins including gelsolin in oral cancer progression has not been investigated before. The functional role and genetic regulation of gelsolin are still not clear. Moreover, genetic defect in betel quid-associated OSCC in Taiwan population are distinct from those in the western populations. This study aims to investigate the role of gelsolin expression in the cellular apoptosis and proliferation, as well as cellular motility and invasion in the cell line models via genetic and epigenetic manipulation of the protein expression.
IV. 研究方法

Transfection and generation of stable cell lines

A total of three expression constructs were evaluated in this study: the pc6-GSNw, the pc6-RGSNn, and the pINDGSN. The pc6-GSNw was derived from subcloning of full length cytoplasmic gelsolin cDNA obtained from Dr. Kwiatkowski in vector LKCG to a new expression vector- pcDNA6-V5/His (Invitrogen Inc., USA) and was established and sequence verified previously. The pc6-RGSNn drive the expression of a ribozyme construct spanning the nt192 to nt198 of gelsolin cDNA previously designed by Yang BT and Shieh DB, et al. OECM-1 cells were transfected with pc6-GSNw using Fugene 6 reagent (Roche Diagnostics GmbH, Germany) according to the manufacture’s instructions and the stable transfected lines were established from individual clone after four weeks of selection in the presence of 10µg/ml Blasticidin (Blasticidin S, hydrochloride, CALBIOCHEM) in the culture medium. pcDNA6-V5/His was used as the vector control. Stable transfectants were maintained in the presence of 10µg/ml of Blasticidin (Blasticidin S, hydrochloride, CALBIOCHEM, USA) for 2 weeks. Individual colonies were picked out and amplified. Three positive colonies were attained, and two of them were failed during expansion before on going the functional studies.

The ecdysone mammalian inducible expression system

The full-length gelsolin cDNA fragment was cloned into the inducible expression vector pIND by the NheI and EcoRV restriction enzyme digestion and ligation. The resulting construct was sequence verified. The construct was cotransfected with pVgRXR into OC-2 cells and the stable transfectants were selected with 250µg/ml G418 (GIBCO BRL) and 25µg/ml Zeocin (Invitrogen). After transfection, the cells were treated with ponasterone A, a steroid hormone ecdysone analog, to activate the expression of gelsolin in a dose-dependent manner via a heterodimeric nuclear receptor (VgEcR and RXR) provided from pVgRXR. In this study, the dose of 3 µM of ponA for 48 hours
was used to induce the gelsolin expression in the following study.

**Epigenetic regulation of gelsolin expression**

Trichostatin A (TSA, sigma), which is a potent histone deacetylase inhibitor, was solved in PBS and diluted to the desired concentration in growth medium in order to investigate modulation of gelsolin expression. For treatment with TSA, OC-2 and OEClM-1 cells were grown on the 10cm culture dishes, switch to the medium containing 5, 10µM TSA for 24 or 48 hours. The protein samples were unable to collect due to prominent cell death in OC-2 line treated with 10µM TSA.

For the induction of demethylation, 5-azacytidine (5-aza) treatment was performed and the 6-azacytidine (6-aza) was served as the control. OEClM-1 or OC-2 were trypsinized and seeded onto 10-cm culture dishes on day 0. The cells were exposed to 10µM of 5-aza or 6-aza for 24 h each on day 2 and day 5 before the collection of the protein lysate on day 8 for further analysis (Veigl et al., 1998).

**Western blot analysis**

To collect the total proteins in the cell lines, cells were expanded to about 70% confluence in a 10cm tissue culture dish, rinsed twice with PBS solution, followed by RIPA buffer protein extraction on ice. Protein concentrations in each cell lysates were measured spectrophotometrically using the Bio-Rad Dc Protein Assay (based on Lowry method) in the SmartSpectTM 3000 (Bio-Rad, Hertfordshire, England).

Fifty micrograms total protein of each cell lysate was loaded on to a 10% SDS-polyacrylamide gel and separated by electrophoresis along with the standard molecular weight marker (Amersham, UK). The gel was transferred to PVDF membranes, blocked overnight and probed by antibodies against gelsolin (2C4; Sigma, USA) and beta-actin (Anti-beta-actin, CHEMICON International, Inc).
**The Invasion assay and the migration assay**

The invasion assay of tumor cells was performed using Transwell™ cell culture chamber (Coring Costar No. 3422, Cambridge, MA, USA) according to methods reported previously (Tsuchiya et al., 2001). Ten µg/ml mitomycin was applied to the culture medium for 2 hours after confluence of the cells to inhibit DNA synthesis. The cells were detached from the flask with 0.25% trypsin-EDTA treatment and resuspended in RPMI with 1% fetal bovine serum at a concentration of 1x10⁶ cells/ml. The cell suspension (100µl) was added onto the upper compartment of chamber. The medium containing 10% serum and 5µg/ml fibronectin (Sigma, USA) was placed in the lower compartment of chamber as the chemoattractant and the invasion chamber sets were incubated for 6 hours at 37°C in the 5% CO₂ incubator. The migration assay was performed using the same system except that the filters were not coated with the Matrigel. The filters were then fixed and stained with 0.5% crystal violet in 20% methanol for an hour then counted under microscope and further quantified by colorimetric method. The crystal violet dye retained on the filters was extracted with 30% acetic acid and colorimetrically assessed by measuring its absorbance at 590 nm.

**Time-lapse videomicroscopy**

The cell migration of the control and transfected cell lines were cultured in a 6cm culture dish in an environmental chamber maintained at 37°C with conditional air flow containing 5% CO₂ and controlled humidity. The chamber was placed on an inverted microscope (ZEISS AXIOVERT135, Germany) equipped with time-lapse image recorder. The migration of cells was recorded every 5minutes for 24 hours under 40x objective power in the phase contrast mode. To calculate the speed of cellular migration, 16 cells were randomly selected from the imaging field and analyzed using a cell tracking software (Imaging tool, USA). The speed was calculated by averaging of migration velocity reference to the previous image frame (µm/min).
**WST-1 proliferation assay**

The cell proliferations were analyzed by WST-1 colorimetric assay. The OECM-1 transfectants in their Log-phase cell cultures were harvested with 0.25% trypsin-EDTA and re-suspend in RPMI with 10% FBS at a concentration of 1x10^5 cells/ml. The cell suspension (100µl) was seeded into a 96-well culture dish and incubated for 1 day. The culture medium was remove from each well and was gently washed with PBS twice before the addition of 100µl/well RPMI with 10% FBS and 10µl/well WST-1 reagent (Roche, USA). After 2 hours of incubation, the cell activities were measured colorimetrically by optical absorbances (450/630nm) using an ELISA plate reader.

**BrdU (5-bromo-2'-deoxyuridine) incorporation assay**

Since cellular proliferation requires DNA synthesis, monitoring of DNA synthesis could both served as another indirect parameter for cell proliferation as well as the cell cycle control and DNA replication activity itself. [\(^{3}\text{H}\)]-thymidine incorporation assay was the most widely and traditionally applied methods to label DNA of the mitotic active cells. Recent development has been able to replace the [\(^{3}\text{H}\)]-thymidine by 5-bromo-2'-deoxyuridine (BrdU). This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. After their incorporation, BrdU is detected by immunoassay using monoclonal antibodies specific for BrdU. The assay in the following study was performed according to the manufacture’s instructions (Roche, Germany). Briefly, 5x10^3 cells were seeded to the 96-well microtiter plate and incubated for 24 hours. Cells were fixed to denature the DNA for improving the accessibility of BrdU incorporation. The anti-BrdU-peroxidase conjugated antibody was bound to the newly synthesized DNA on the BrdU incorporated sites. The chromogenic substrates tetramethylbenzidine (TMB) were then converted by the antibody conjugated peroxidase and detected by measuring the absorbance at 450nm with a reference wavelength at 650 nm using an
ELISA reader. The developed color and thereby the absorbance values were directly correlated to the amount of DNA synthesis and the number of proliferating cells.

**Cell growth assay**

Cell growth represents the summed effects of cell proliferation and cell death. To determine the cell growth rate of OECM-1 cells and gelsolin overexpressing transfectants, 5x10⁴ cells were plated in 60 mm diameter culture dishes. The cultures were maintained for 1, 2, 3, 4, 5 days before harvest for differential quantification of viable and dead cells. The viable and the dead cells were identified using trypan blue (Biological Industries, USA) exclusion assay. The numbers of viable (trypan blue-negative) and dead (trypan blue-positive) cells were counted with a hemocytometer and were recorded for statistic analysis.

**FACS analysis**

For FACS analysis, cells were harvested by trypsinization, washed in PBS, and fixed in ice-old filtered 70% ethanol for overnight at 4°C. After adding 5 ml ISOTONE II (Level, USA), the cell suspensions were gently mixed then centrifuged at 3000 rpm for 10 minutes. The pallets of the cells were resuspended with 0.4 ml ISOTONE II, and then filtered with 80 µm nylon mesh into FACSort tubes (Falcon). 100µl of 400µl/ml propidium iodide (PI) and 1 mg/ml RNase were added into each tube for DNA staining and for eliminating RNA interference, respectively. The reaction mixture was incubated overnight at room temperature. The analysis was performed in a Beckton-Dickinson FACSCAN using CellQuest software.

**Statistic analysis**

The statistic analysis was carried out by the Jump statistic software system (SAS, USA). A crosstabulations of the expression profile to the disease category (premalignant lesion, primary oral cancer and metastatic cancer) and clinicopathological factors (invasion status, tumor size and the
age of disease onset) was performed. The correlation of gelsolin expression and the disease category as well as the clinical pathological parameters was first analyzed by $\chi^2$ tests for the significance in each pair of variants. To analyze the prognostic significance of gelsolin expression, Kaplan-Meier survival analysis was performed for the effect of tumor gelsolin expression level to the five-year disease-free survival in each patient group.

Trend test was performed to evaluate the dosage association between gelsolin and involucrin expression in the tissue and oral cancer cell cultures. For all analyses, P value of less than 0.05 was accepted as statistically significant.
V. 結果與討論:

1. **Inhibition of cell growth and proliferation in gelsolin-expressing OECM-1 cells**

   Gelsolin was reported to be downregulated in many malignancies, and also in OSCC as demonstrated in this study. When transfected gelsolin in to oral cancer cell line, OECM-1, gelsolin overexpressing OECM-1 (OECM1-gsn6) showed a slower growth rate in comparison with the wild-type and the vector-only cells. To examine the cell growth curve, the same numbers (10^4) of cells were seeded into 6-cm dish. Cells were trypsinized and counted on day 2, 4, 6, 7, 8. Gelsolin expression suppressed the growth rate of cells. (Figure 13) However, cell growth was the results of cell death and proliferation. WST-1 assay, which measures the number of metabolically active cells, found that gelsolin expression showed no effect or little enhancement in the cell proliferation (OECM-1 v.s gsn6, p= 0.076; Figure 14). Another method, BrdU incorporation assay, was applied to verify the cell proliferation. Since replication of cellular DNA is required for cell proliferation, the monitoring DNA synthesis is another indirect parameter of cell proliferation as well as being suitable for study of the regulation of DNA synthesis. OECM1-gsn6 cells inhibited cell proliferation compared with the wild type and the vector control cells (p= 0.0002 and 0.0003; Figure 15). Cell death was detected with trypan blue exclusion method. Compared with vector control OECM1-pc6 cells, OECM1-gsn6 cells (gelsolin-overexpression) had higher percentage of dead cells among their total cell population, but no differences in the number of dead cells indicating a more crucial influence from cell proliferation rather than apoptosis (Figure 16). Flow cytometry also revealed a slightly increased cell apoptosis in OECM1-gsn6 (p= 0.11). Suppression of cell growth in OECM1-gsn6 cells mainly resulted from restraining cell proliferation by reduced DNA replication rate (maybe lesser influence on apoptosis).

2. **Promotion apoptosis of gelsolin expression in response to apoptosis induction**

   To determine the effect of gelsolin overexpression on apoptosis, OECM-1 cells transfected
with the gelsolin expression construct and the control vector, were deprived of serum for 48 hours to induce apoptosis. Apoptotic cells were stained with propidium iodide and counted by FACSCAN. Gelsolin expressions in OECM-1 cells (OECM1-gsn6) presented slightly enhanced cell death in comparison with the vector control cells (OECM1-pc6) \( (p=0.001) \). After apoptosis induction by serum deprivation, the percentage of apoptotic cells in OECM1-gsn6 showed 2-fold increase - significantly promoted apoptosis response compared with OECM1-pc6 cells (Figure 17). The cell numbers of OECM1-pc6 and its wild-type cells were decreased in G2/M phase suggesting most of the cells were under cell cycle arrest after 48-hour serum deprivation (Figure 18). Another apoptosis induction mechanism, the anoikis, was employed in OECM-1 model to evaluate the influence of gelsolin expression in the process. Significant number of cells ongoing apoptosis on the second day after anoikis induction. However, unlike serum deprivation, anoikis induced apoptosis failed to show significant association with gelsolin expression (Figure 19).

3. Effects of gelsolin expression on cellular invasion and motility

In the above IHC observation, the gelsolin expression levels in cancer lesions were found to be related with the invasive phenotype. Therefore, \textit{in vitro} invasion and migration assays were performed to further study the functional role of this molecule in such processes. Matrigel represented the reconstituted basement membrane components were used to evaluate the ability of OECM-1 and their transfectants to invade through the matrix and transmigrate from the upper compartment of Transwell cell culture chamber to the lower surface in the presence of chemoattractant - 5µl/ml fibronectin. Gelsolin significantly promoted invasion of OECM-1 cells through Matrigel onto the lower surface of the filter membranes \( (p=0.019; \text{Figure 20}) \). In motility assay, two different methods were used to evaluate the effect of gelsolin expression in OECM-1 cell line model. In the chamber assay, the cells were seeded into the upper compartment of Transwell chamber without precoated with Matrigel on the filters and 5µl/ml fibronectin as a chemoattractant. Gelsolin expression has a slight inhibition in OECM-1 cellular migration through the Transwell.
chambers. (p = 0.266; Figure 21) Videomicroscopy was used to further identify the dynamic effect on cell motility affected by gelsolin expression. The results were consistent with the chamber assay in that gelsolin expression suppressed cell migration (p = 0.015; Figure 22).

4. **Epigenetic regulation of gelsolin expression in oral cancer cells**

Trichostatin A (TSA), an inhibitor of histone deacetylase, treatment increased gelsolin expression in bladder, cervical, colon and breast carcinoma cells, implying that histone acetylation can play a regulatory role. To determine if TSA upregulated gelsolin expression in human OSCC, we analyzed gelsolin protein levels in OC-2 and OECD-1 cells to expose to 1, 10µM TSA for 24 hours. The cell death of OECD-1 cells was increased with increasing TSA dosage, and western blot analysis showed that the expression of gelsolin was raised in a dose dependent manner (Figure 23, 24). 1µM TSA treatment in OC-2 cells did not significantly induce cell death. When increase TSA concentration to 5 and 10µM, large amount of cells were dead. In OC-2 cells, upregulation of gelsolin was observed in the presence of more than 5µM TSA. Gelsolin expression was unable to quantify under 10µM TSA treatment due to excessive number of cell death for the collection of sufficient amount of cell lysate (Figure 24A). OECD-1 cell was more sensitive to TSA induction of gelsolin expression. One µM TSA was able to induce gelsolin overexpression to the plateau level in 24 hours (Figure 24A, B).

The extent morphological alterations upon TSA treatment presented dose dependent manner. In OC-2, the cells became round up or like tear-drop morphology and dissociated from each other (Figure 23 A). In OECD-1 cells, TSA treatment converted the cell morphology from squamous to spindle shaped and encouraged the extension of cellular processes. Detachment from culture dish and round up of the cells were not common as in the OC-2 line although the same phenomenon of reduced cell to cell contact was noted in both lines.

Increased expression levels of involucrin were also observed upon TSA induced gelsolin expression suggesting that TSA may induce cell differentiation in the meantime or gelsolin
participate in the differentiation process.

5-azacytidine (5-aza) is a potent demethylation agent. Although previous studies have been able to induce gelsolin expression via demethylation strategy in other types of cell lines, this study failed to induce gelsolin expression by 5-AZA treatment in the given dosage of 10µM concentration in both lines (Figure 25). The results indicated that DNA demethylation might not be able to enhance gelsolin expression in oral cancers.
Discussion

Previous studies have demonstrated that gelsolin is down regulated in many cell types of human malignancies, such as breast, lung, gastric, colon, bladder, prostate carcinomas, etc (Asch et al., 1996; Asch et al., 1999; Chaponnier and Gabbiani, 1989; Dosaka-Akita et al., 1998; Furuuchi, 1997; Kuzumaki, 1996; Lee et al., 1999; Tanaka et al., 1995; Zhang et al., 1997). It has also been shown to be one of the most significant genetic defects in genome wide scanning by SAGE analysis (Zhang et al., 1997). In this study, we continue our previous observation of a biphasic expression profile of this protein during oral carcinogenesis and began to explore its functional role using oral cancer cell line model and gene transfer strategies.

Tanaka et al, Furuuchi et al, and Sagawa et al indicated that introducing wild type gelsolin cDNA suppressed tumorigenicity in human bladder, colon, and lung cancer cell lines (Furuuchi, 1997; Tanaka et al., 1999). Our previous study failed to establish the stable clone of overexpressed wild type gelsolin in OC-2 cells. The overexpression of gelsolin in OC-2 line promoted the “round-up” and “tadpole” morphological transformation and significantly suppressed cellular growth. Although introducing full length cytoplasmic gelsolin cDNA was succeeded in another oral cancer cell line OECM-1. The derived OECM1-gsn6 cells presented significant subdued cell growth rate as compared with vector control OECM1-pc6 cells and parental OECM1 cells. The growth rate of cells is the balance of cell death and cell proliferation, so the cell regression was owing to restrained cell proliferation or enhanced the apoptotic process. Apoptotic rates assayed either by counting the dead cells or evaluate by PI staining using flow cytometry were no difference within OECM1 transfectants and parental cells, suggesting that increasing intrinsic apoptotic cells in OECM1-gsn6 may not play a major role in their growth retardation. Besides, OECM1-gsn6 cells promoted cell apoptosis in response to serum deprivation for 48 hours while no significant effect to anoikis induced apoptosis. Controversial results were existed in the apoptosis or anti-apoptosis function of gelsolin in previous studies. In Jurkat cell model, a lymphoblastoid T cells, the authors suggested that gelsolin inhibited apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release (Azuma et al., 2000; Koya et al., 2000; Kusano et al., 2000; Ohtsu et al., 1997).
Another study suggested that gelsolin in complex with PIP2 inhibited caspase-3 and -9 to retard apoptotic progression. These implied other unknown gelsolin associated apoptosis regulatory pathways in may involve in epithelial cell type.

In cell proliferation, the WST-1 assay, which measured the enzyme activities of mitochondrial dehydrogenases of viable cells, was used to evaluate the number of metabolically active cells. Contrary, a slightly elevated cell vitality was observed in OECM1-gsn6 cells instead, despite the retarded overall growth rate in this line compared to the control and wild type lines. Another method was applied to assess the cell proliferation by monitoring DNA synthesis - the BrdU incorporation assay, which is based on the incorporation of the pyrimidine analogue BrdU into the DNA of proliferating cells. OECM1-gsn6 significantly inhibited cell proliferation measured by BrdU incorporation. Two distinct results of cell proliferation by different methods may be explained by that the higher mitochondrial activities in OECM1-gsn6 masking the assay result from the inhibited DNA synthesis regardless the alterations in the number of viable cells observed at 24 hours after seeding the cells. Previous studies have shown that the cell viability not necessarily associated with the BrdU incorporation assay. The two assays address different pathways describe the cell proliferation activity. The E1A oncoprotein induce DNA synthesis and apoptosis in cardiate myocytes. (Liu and Kitsis, 1996) Cells treated with actinomycin D inhibited DNA synthesis but not the metabolic activity of the cells. In the presence of low bacterial cell ratio, Helicobacter Pylori reduced the number of epithelial cells but increase the rate of DNA synthesis. (Wagner et al., 1997)

Sagawa N et al revealed that gelsolin inhibit cell proliferation in a human lung cancer cell line through suppressing the activation of protein kinase C involved in phospholipid signaling pathways (Sagawa et al., 2003). The data suggest that gelsolin induced growth suppression was primarily due to inhibition of DNA synthesis rather than enhancing the apoptosis and elevated gelsolin have little or no effect on the metabolic activity of the oral cancer cell in normal culture condition. However, in the presence of environmental stress, gelsolin expression promoted apoptosis in response to serum deprivation but not anoikis. These findings may explain the diminished gelsolin expression during oral carcinogenesis in our clinical study. The molecular pathway in the regulation of cell cycle control, DNA
synthesis, and susceptibility to apoptosis induction should be interest for future investigation.

Gelsolin is able to disrupt the actin cytoskeleton by virtue of its F-actin severing activity, while, on the other hand, it promoted actin polymerization by enhancing the nucleation in the target regions (Kwiatkowski, 1999). Overexpression of gelsolin in fibroblasts caused a reduction in filamentous actin and displayed enhanced cell motility rate. However, cells expressed wild type gelsolin reduced chemotactic migration toward fibronectin and suppressed lung colonization in spontaneous metastasis assay in B16-BL6 mouse melanoma cells (Fujita et al. 2001). De Corte et al mentioned that gelsolin overexpressing MDCK cells slightly inhibited wound healing-induced cell motility (in their unpublished data). Therefore, whether the increased gelsolin expression in cancer progression through precancers to primary or metastastic cancers was due to enhancing cell motile activity still needs to be validated in the functional assays. In this study, gelsolin-transfected OECM-1 (OECM1-gsn6), unlike OC-2, showed no significant cell morphological change. Also, OECM1-gsn6 cells presented the same or reduced chemotactic migration towards fibronectin concentration gradient using Transwell chamber and timelapse videomicroscopy in comparison with vector control cells (OECM1-pc6). Fujita et al revealed that gelsolin expressing in B16-BL6 mouse melanoma cells suppressed cell chemotactic migration. Gelsolin may therefore affect cell motility differently in oral epithelial cells and fibroblasts. Promotion of Matrigel invasion was observed in OECM1-gsn6 cells. Overexpressing gelsolin in MDCK cells also supported the finding of gelsolin-induced epithelial cell invasion, and the author demonstrated that gelsolin expression inhibited E-cadherin-mediated cell aggregation without altering E-cadherin-β-catenin-α-catenin complex formation. The data could partially explain the conversion of a non-invasive to an invasive tumor may depend on acquiring certain level of gelsolin expression. However, this speculation should be further investigated by regulating protein levels in cell line model using inducible system. One interesting finding is that loss or decreased cellular adhesion was observed in gelsolin overexpressed lines after thawing from liquid nitrogen for culture (data not shown). The effect of gelsolin expression to cellular adhesion may be another important factor to be investigated. Invasive carcinoma cells often lack adherence junctions and desmosomes, resulting in a loss of polarized structure characteristic of epithelium cells. There’s no report dealing with the integrity, strength or the
spatial distribution or numbers of adhesion plaques in the cells after alterations of cytoplasmic gelsolin via genetic or epigenetic manipulations. Besides, no reports have investigated the effect of gelsolin expression on MMP (matrix metalloproteinases) or other extracellular matrix enzyme expressions in the cancer cells. The enhanced invasion ability while not cellular motility may also associated with enhanced digestion of extracellular matrix of MMP and downregulating tissue inhibitors of MMP (TIMPs) and anoikis inhibition (Oxford and Theodorescu, 2003). In this study, we did not observe the inhibition of anoikis and enhance in cell motility, so other processes associated with cancer cells invasion remained to be determined. To conclude, gelsolin expression in OECM-1 cells did not alter the cell motility, but significantly promote cellular invasion into Matrigel.

TSA treatment of OECM-1 and OC-2 cells both upregulated gelsolin and involucrin expression and caused significant cell apoptosis, consistent with previous reports. This suggested that epigenetic mechanisms may be involved in the regulation of gelsolin expression in cancer cells. Evidence in support of epigenetic mechanisms regulating expression of gelsolin has been reported in human breast cancer cells, demonstrating epigenetic changes in chromatin structure lead to downregulation of gelsolin and hypoacetylation of histones rather than hypomethylation of regulatory CpG regions in promoter appear to the more important in the repression (Mielnicki et al., 1999). Other studies also supported different histone deacetylase inhibitors, sulfonamide, sodium butyrate, oxamflatin, MS-275, apicidin, and etc in human cancer cell lines (Fournel et al., 2002; Han et al., 2000; Kim et al., 1999; Mielnicki et al., 1999). Jeung-Whan et al (Han et al., 2000) showed that apicidin inhibited proliferation and cell morphological changes of tumor cells via induction of p21WAF/Cip1 and gelsolin. These small molecular inhibitors of HDACs have emerged as antiproliferative agents because of their ability inhibit proliferation, induce apoptosis, and/or cell differentiation. It is believed that induction of p21WAF/Cip1 and blockade of tumor cell cycle progression are critical for antitumor activities of all the HADC inhibitors. To conclude, gelsolin is the first example of a tumor suppressor gene downregulated in human OSCC by changes in histone acetylation in this study.
References


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Figures

Figure 1

Figure 2

Figure. Cell growth curve. Gelsolin over-expression suppressed cell growth in OECM-1 cells (OECM1 g6).
Figure 3

Effect of gelsolin on invasion of OECM-1 cells into Matrigel. The filters of Transwell chambers had been precoated with 100 μl of 1 mg/ml Matrigel. Tumor cells (10^5) were added to the upper compartment of Transwell chamber with 5 μg/ml fibronectin in the lower chamber as a chemoattractant. After 6-hour incubation, invaded cells were counted under a light microscopy. Each value represented the mean (±SE) of three independent experiments.

Figure 4

Videomicroscopy observation of gelsolin overexpressing OECM-1 cells. The cells were recorded every 5 minutes for 2 hours in 37°C with 5% CO₂, which mimic the environment in the cell culture incubator. The migration routes of cells were tracked by a software, and ten cells were randomly sampling in a field for this experiments. Each value represented the mean (±SE).
Figure 5. Effect of gelsolin on migration of OECM-1 cells. Tumor cells \((10^5)\) were added to the upper compartment of Transwell chamber with 5 \(\mu\)g/ml fibronectin in the lower chamber as a chemotactagent. After 6-hour incubation, invaded cells were stained with 0.5% crystal violet in 20% methanol for 30 minutes. The stained cells were colorimetrically assessed by measuring their absorbance at 590 nm. Each value represents the mean (±SE) of three independent experiments.

Figure 6. WST-1 assay. OECM-gsn6 had a slightly increase on metabolic activity.
Figure 7

BrdU incorporation assay. OE CM-gsn6 had a significant slower proliferation and DNA synthesis rate, compared with parental and vector control cells.

Figure 8

Serum deprivation-induced apoptosis. OE CM-gsn6 promoted cell apoptosis in response to serum deprivation for 48 hours, in comparison with parental and vector control cells.
Figure 9

Figure 8

Figure 10

Figure. Anoikis (Homeless cell death) induced apoptosis. There was no difference between three cells when anoikis induced for 8, 12, 24 hours.

Figure. Cell morphology for OC-2 (A) and OECM-1 (B) treated with different dose (0, 1, 10 nM) of trichostatin A. Cell death increased with adding higher dose of TSA in OECM-1 cells, but serious cell death of OC-2 was observed at the level of 10nM.
Figure 11

Figure. Western blot analysis of gelsolin and involucrin expression after TSA treatment in OC-2 and OECM-1 cells. (A) Different doses of TSA were treated as above each lane. (B) OECM-1 cells were treated with 1 or 5 μM TSA for 24 or 48 hours.
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