MOLECULAR STUDIES OF ONCOGENES *JK-1* AND *GAEC1*, CISPLATIN-RESISTANCE AND TUMOR INVASION IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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Ph.D

The Hong Kong Polytechnic University

2016

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Molecular Studies of Oncogenes *JK-1* and *GAEC1*, Cisplatin-resistance and Tumor Invasion in Esophageal Squamous Cell Carcinoma

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A thesis submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy

October 2015

CERTIFICATE OF ORIGINALITY

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Miss Chan Dessy

ABSTRACT

Esophageal squamous cell carcinoma accounts for 80% of esophageal cancers worldwide. It was characterized by great geographical variation in incidence and high mortality rate. Although many therapeutic strategies have been adopted, the survival rate is still poor. Therefore, better understanding of pathogenesis, development of chemo-resistance as well as discovery of novel therapeutic approaches are essential.

In the study of a novel oncogene *GAEC1*, reduction in proliferation rate and increase in apoptotic population were observed with effective suppression of *GAEC1* expression on ESCC cell line KYSE150 by *GAEC1* targeting siRNA expression vector. Calpain 10 (*CAPN10*) was identified as the downstream regulatory gene of *GAEC1*. Immunohistochemical (IHC) staining of CAPN10 on tissue microarray (TMA) showed that low expression of CAPN10 predicted poor survival in ESCC patients.

Target-suppression of another novel oncogene *JK-1* on ESCC cell line KYSE70 was demonstrated using a *JK-1* targeting siRNA expression vector. KYSE70 cells with suppressed *JK-1* expression showed reduction in cell proliferation rate, decrease in cell migration rate, loss of anchorage-independent

growth and acquisition of growth inhibition. For the IHC study of JK-1 expression, 14 out of 16 (87.5%) cases with pre-malignant lesions belonged to JK-1 high expression group which was less commonly found in tumor (7/26; 26.9%), suggesting that JK-1 expression may be more crucial at the pre-malignant stage during the early phase of tumor formation.

For the study about the changes in gene expression profile in cisplatin (CDDP)-resistant ESCC tumor cells, a CDDP-resistant ESCC cell line, SLMT-1/CDDP1R, with 2.8-fold increase in resistance to cisplatin compared with the parental cells was established from SLMT-1 cells. cDNA microarray analysis revealed that *IGFBP5* showed the highest level of down-regulation in SLMT-1/CDDP1R cells compared to SLMT-1 parent cells. Suppression of *IGFBP5* mediated by *IGFBP5*-targeting siRNA in parental SLMT-1 cells confirmed that *IGFBP5* suppression was one of the mechanisms for ESCC cells to acquire CDDP-resistance. More importantly, up-regulation of *IGFBP5* using *IGFBP5* expression vector sensitized SLMT-1/CDDP1R cells to cisplatin.

For the study about the anti-cancer effect of a novel synthetic quinoline-based compound 91b1, cytotoxic effect on the five ESCC cell lines (KYSE150, KYSE450, KYSE510, KYSE30 and HKESC-4) was detected using MTS cytotoxicity assay with the MTS_{50} ranging from 1.80 -10.23μg/mL. The anti-

cancer effect of 91b1 was also comparable to the front-line chemotherapeutic drug CDDP and showed 1.6 and 2.1 times lesser cytotoxic effect to non-tumor cell lines NE-3 and HEK293 than CDDP. cDNA microarray analysis on the 91b1-treated (9.5μg/mL) KYSE150 cells compared with the parental cells identified Chemokine (C-C motif) Ligand 5 (*CCL5*) as the most significantly downregulated target. By qPCR analysis, IHC staining of CCL5 and CCL5 specific ELISA, CCL5 was shown to be suppressed in mRNA and protein expression with 91b1 treatment in a dose-dependent manner. Up-regulation of CCL5 was also detected in 87.5% (7/8) of untreated ESCC cell lines by IHC analysis compared to non-tumor esophageal epithelial cell line NE-3. IHC analysis on ESCC specimens also revealed that upregulation of CCL5 was more commonly found in ESCC tumor and pre-malignant tissues, of which 20 out of 26 (76.9%) tumor specimens and 10 out of 16 (62.5%) specimens with pre-malignant tissues belonged to CCL5 high expression group. The trans-well matrigel invasion assay also demonstrated that CCL5 protein could enhance the invasion ability of KYSE30 cells compared with the untreated cells. The results implied that 91b1 could effectively induce cytotoxicity on ESCC cells and the mechanisms may involve the downregulation of CCL5 expression and suppression of tumor invasion.

The overall results of the present study thus provided the possible novel

therapeutic directions for the treatment of ESCC at molecular and pharmaceutical levels. The findings on the anti-cancer actions of the novel compound 91b1 and the approach for reversing the CDDP-resistance phenotype hopefully can be further extended to other tumor types in future.

PUBLICATIONS

Journal Articles

A. Published journal article directly related to this thesis

Oncogene *GAEC1* regulates CAPN10 expression which predicts survival in esophageal squamous cell carcinoma

Dessy Chan, Miriam Yuen-Tung Tsoi, Christina Di Liu, Sau-Hing Chan, Simon Ying-Kit Law, Kwok-Wah Chan, Yuen-Piu Chan, Vinod Gopalan, Alfred King-Yin Lam, Johnny Cheuk-On Tang
World Journal of Gastroenterology. 2013 May 14; 19(18): 2772–2780.

B. Published journal article not directly related to this thesis

Synthesis of 8-hydroxyquinoline derivatives as novel anti-tumor agents.

Sau Hing Chan; Chung Hin Chui; Shun Wan Chan; Stanton Hon Lun Kok; **Dessy Chan;** Miriam Yuen Tung Tsoi; Polly Hang Mei Leung; Alfred King Yin Lam; Albert Sun Chi Chan; Kim Hung Lam; and *Johnny Cheuk On Tang

ACS Medicinal Chemistry Letters, v. 4, (2), 2013, Feb, p. 170-174

C. Submitted journal article not directly related to this thesis

Anti-cancer effects of a quinoline derivative 83b1 through targeting the peroxisome proliferator-activated receptor δ and suppressing cyclooxygenase-2 expression in human esophageal squamous cell carcinoma (ESCC)

Ivan Ho Yuen Pun, **Dessy Chan**, Sau Hing Chan, Po Yee Chung, Yuan Yuan Zhou, Simon Law, Alfred King Yin Lam, Albert Sun Chi Chan, Chung Hin Chui, Kim Hung Lam, Johnny Cheuk On Tang

Accepted by Cancer Research and Treatment

D. Manuscripts under preparation (directly related to this thesis)

Characterization of the anti-cancer effect of a novel quinoline-based compound 91b1 with suppression of chemokine (C-C motif) ligand 5 (CCL5) in human esophageal carcinoma

Dessy Chan, Ivan Ho Yuen Pun, Sau Hing Chan, Po Yee Chung, Yuan Yuan Zhou, Simon Law, Alfred King Yin Lam, Albert Sun Chi Chan, Chung Hin Chui, Kim Hung Lam, Johnny Cheuk On Tang

Submitted to Cancer Research and Treatment

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and thanks to my project supervisor, Dr. Johnny Tang Cheuk-on, who provided me with enthusiastic supervision, supportive guidance and valuable suggestions throughout the period of my research project.

I am also grateful to the members of Dr. Tang's research team, including Miss Yan Wong, Miss Kathy Hiu-Ying Poon, Ms Cyin Ng, Ms Phoebe Wong, Miss Miriam Yuen-Tung Tsoi, Mr. Ivan Ho-Yuen Pun, Miss Po-Yee Chung, Miss Yami Yeung, Mr. Yannick Ying-Lung Hung, Miss Yuan-Yuan Zhou for their kindly assistance on laboratory work and sharing of their own valuable research experience.

I also would like to thank Dr. Penny Sau-Hing Chan for the synthesis of the novel quinoline derivative 91b1.

Special thanks are given to Dr. K. W. Chan from the Department of Pathology of The University of Hong Kong for his kind assistance in reviewing the slides for the immunohistochemical studies, and to Prof Simon Law from the Department of Surgery of the University of Hong Kong for his generous help in providing the clinicopathological information for the present study.

I would like to extend my sincere thanks to all laboratory technicians, including Mr. Chor-hing Cheng, Miss Echo Wan, Miss Sarah Yeung, Mr. Man-Cheung Leung and Mr. Lap-Yung Cheung from the Department of Applied Biology and Chemical Technology of The Hong Kong Polytechnic University and Mr. Paul Wong from Department of Pathology of The University of Hong Kong for their assistance on the histopathology work.

Special thanks are also given to my family and friends, especially Mr. Yiu-Nam Chan, Ms Ti-Sin Ju, Mr. Eddy Tin-Ho Chan, Mr. Cambo Chin-Chung Ho, Ms Joyce Qing-Yan Luo, Miss Suqing Tan, Mr. Stephen Kim, Mr. Wing-Yeung Liu and Miss Jill Shuk-Ying Ng for always giving me support and encouragement.

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LIST OF ABBREVIATIONS

91b1	: 5,7-dibromo-1,2,3,4-tetrahydro-2-methylquinolin-8-ol
ATCC	: American Type Culture Collection
BCH	: Basal cell hyperplasia
CAPN10	: Calpain 10
CCL5	: Chemokine (C-C motif) ligand 5
CDDP	: Cisplatin; cis-diamminedichloroplatinum (II); cis-diammineplatinum (II) dichloride
DMEM	: Dulbecco's modified Eagle's medium
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleoside triphosphate
EADC	: Esophageal adenocarcinoma
EC	: Esophageal cancer
ELISA	: Enzyme-linked immunosorbent assay
ESCC	: Esophageal squamous cell carcinoma
ESD	: Esophageal squamous dysplasia
et al.	:*et alia*
FBS	: Fetal bovine serum
GAEC1	: Gene amplified in esophageal cancer 1
IGF	: Insulin like growth factor
IGFBP5	: Insulin-like growth factor binding protein 5
IGF-R	: IGF-receptor
IHC	: Immunohistochemistry
KSFM	: Keratinocyte serum-free medium
MTS	: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
p	: p value
PBS	: Phosphate buffered saline
qPCR	: Quantitative polymerase chain reaction analysis
RNA	: Ribonucleic acid
RNAi	: RNA interference
RT-PCR	: Reverse transcription-polymerase chain reaction
siRNA	: Small interfering RNA
TMA	: Tissue microarray

LIST OF ABBREVIATION FOR UNITS

°C	: degree Celsius
bp	: base pair
M	: molar
mL	: milliliter
min(s)	: minute(s)
µL	: microliter
ng	: nanogram
nm	: nanometer
nt	: nucleotides
p	: p value
pg	: picogram
rpm	: revolutions per minute
sec (s)	: second(s)
µg	: microgram
µm	: micrometer

Chapter 1 Introduction & Literature review

1.1. Esophagus

1.1.1. Gross features of esophagus

The esophagus is a hollow muscular tube extends from the upper sphincter to the lower sphincter, connecting the pharynx to the stomach (Jobe, B. A. *et al.*, 2009). It is one of the organs of the digestive system, responsible for delivering food and liquid from pharynx to the stomach. In human, esophagus is about 18 to 26cm long and is lined throughout its length with a non-keratinized stratified squamous epithelium (Ross, M. and Pawlina, W., 2006).

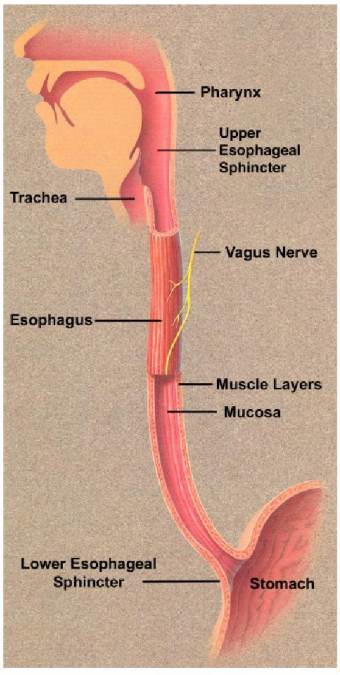


Figure 1.1 A figure of the esophagus showing its location next to the pharynx (Adopted from webpage of Patankar Research group, Northwestern University http://patankar.mech.northwestern.edu/researchfolder/esophageal.htm)

1.1.2. Anatomy of esophagus

Beginning at the hypopharynx, the esophagus lies posterior to the trachea and the heart, passing through the posterior mediastinum and entering the stomach through an opening in the diaphragm called the hiatus (Greene, F. L. *et al.*, 2006). For systematic classification, staging and reporting of cancer, the esophagus is divided into three regions: cervical esophagus, thoracic esophagus and abdominal esophagus (Greene, F. L. *et al.*, 2006; Jobe, B. A. *et al.*, 2009)

1.1.3. Histology of esophagus (Greene, F. L. *et al.*, 2006)

Histologically, esophagus contains four layers: mucosa, submucosa, muscularis externa and adventitia.

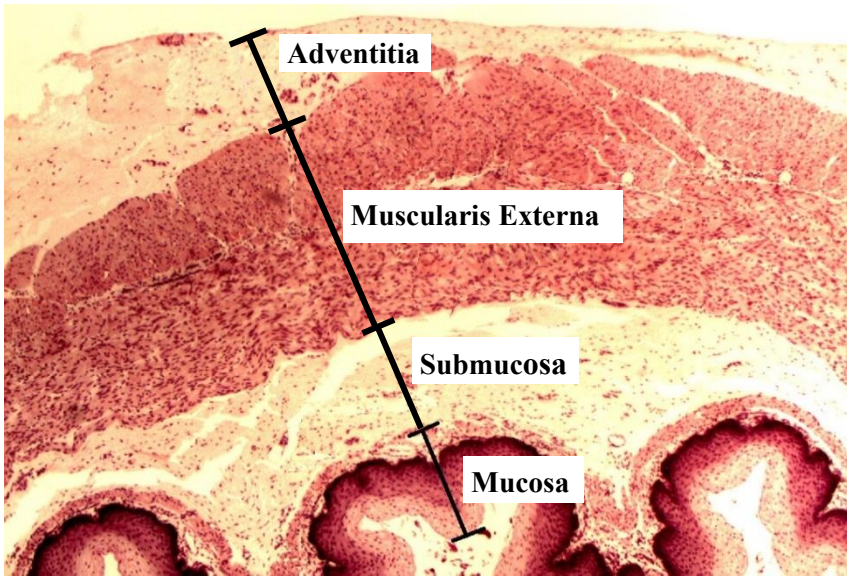


Figure 1.2 The histology of esophagus (H&E, x 20).
(Adopted from Jobe, B., C. Thomas, and J. Hunter, Esophageal Cancer: Principles and Practice. 2009: Demos Medical Pub)

1.1.3.1. Mucosa

Mucosa is the innermost layer of esophagus. It is arranged in longitudinal folds and faces the esophageal lumen, acting as a barrier to separate the esophageal lumen and the body tissues. Mucosa has three sub-layers: mucous membrane, lamina propria and muscularis mucosa.

Mucous membrane is the first sub-layer of mucosa which consisted of non-keratinized squamous epithelium. It covers nearly entire inner surface of esophagus. Lamina propria is a thin connective tissue structure containing vascular structures and mucosa secreting glands (Jobe, B. *et al.*, 2009).

Muscularis mucosa, the deep layer of mucosa, is composed of longitudinally organized smooth muscle that begins near the level of the cricoids cartilage. It is thick in the proximal portion of esophagus and function by assisting swallowing (Ross, M. and Pawlina, W., 2006).

1.1.3.2. Submucosa

Submucosa is a layer of loose connective tissue containing lymphocyte, plasma cells, nerve cells, the vascular network and submucosal glands (Jobe, B. *et al.*, 2009).

1.1.3.3. Muscularis externa

Muscularis externa is composed of two types of muscles: striated muscle and smooth muscle. The upper one third of esophagus is composed of striated muscle. The middle one third have both types of muscle interwoven and the remaining one third consists of smooth muscle only (Ross, M. and Pawlina, W., 2006). Muscularis externa enable the rhythmic muscular movements of esophagus lumen as well as provides mechanical support to the esophageal lumen (Jobe, B. *et al.*, 2009).

1.1.3.4. Adventitia

The adventitia is an external fibrous layer that covers the esophagus. It is composed of loose connective tissue and contains small vessels, lymphatic channels and nerve fiber. It joints the esophagus to the neighboring structure.

1.2. Esophageal cancer

1.2.1. Classification of esophageal cancer

Esophageal cancer can be subdivided into epithelial or non-epithelial (Table 1.1), where esophageal adenocarcinoma (EACD) and esophageal squamous cell carcinoma (ESCC) comprise the overwhelming majority of esophageal cancer that over 90% of esophageal cancers are these two histology subtypes (Enzinger, Peter

C and Mayer, Robert J, 2003; Enzinger, P. C. and Mayer, R. J., 2003; Holmes, R. S. and Vaughan, T. L., 2007; Katzka, D. A. and Metz, D. C., 2003). In Hong Kong, ESCC accounts for 90% of diagnosed esophageal cancer cases that is similar to other Asia regions(Tong, D. and Law, S., 2015; Tse, L. a. *et al.*, 2007). While for the Western regions, EACD is the predominant historical type of esophageal cancer. ESCC and EACD are different in their underlying patterns of incidence and key etiologic factors. However, both these types share high mortality rate (Holmes, R. S. and Vaughan, T. L., 2007).

Table 1.1 Classification of esophageal cancer

(Adopted from Katzka, D., and Metz, D. Esophagus and stomach: Mosby Inc, 2003, p.100.)

Malignant Tumor Type	Clinical behavior
Epithelial -malignant	
- **Adenocarcinoma**	aggressive
- **Squamous cell carcinoma**	aggressive
- **Squamous cell carcinoma with spindle cell component**	slow growing to aggressive
- **Verrucous squamous cell carcinoma**	slow growing to aggressive
- **Adenoacanthoma and adenosquamous carcinoma**	aggressive
- **Adenoid cystic carcinoma**	aggressive
- **Mucoepidermoid carcinoma**	very aggressive
- **Melanoma**	very aggressive
- **Carcinoid tumor**	slow growing
- **Small cell carcinoma**	very aggressive
- **Choriocarcinoma**	aggressive
Non-epithelial - malignant	
Sarcoma	very aggressive
- **Leiomyosarcoma**	
Kaposi's sarcoma	
- **Rhabdomyosarcoma**	
- **Neurogenic sarcoma**	
Lymphoma	varied depending on subtype
- **Hodgkin's disease**	
- **Non-Hodgkin's lymphoma**	

1.2.1.1. Esophageal squamous cell carcinoma (ESCC)

Esophageal squamous cell carcinoma (ESCC) was defined by World Health Organization as a malignant epithelial tumor with squamous cell differentiation, microscopically characterized by keratinocyte-like cells with intercellular bridges and/or keratinization (Jobe, B. *et al.*, 2009). Gross appearance of ESCC varies. However, superficially invasive cancers often have a plaque-like appearance. Verrucous carcinomas are typically cauliflower-like. Invasive tumors may be

ulcerated, fungating, or may show a relatively small mucosal defect but infiltrate deeply into the underlying tissue (Jobe, B. *et al.*, 2009; Katzka, D. A. and Metz, D. C., 2003).

1.2.1.2. Esophageal adenocarcinoma (EADC)

Esophageal adenocarcinoma is defined by a malignant epithelial tumor of the esophagus with glandular differentiation. The tumors typically arise from Barrett's esophagus in the lower third of the esophagus(Jobe, B. *et al.*, 2009).

1.2.2. Epidemiology

Esophageal cancer is the eighth most common cancer in the world. It caused over 450,000 new cases and around 400,000 deaths worldwide (Arnold, M. *et al.*, 2015; Torre, L. A. *et al.*, 2015). ESCC is the more common subtype of esophageal cancer in the world. 80% of global ESCC cases occurred in the Central and South-East Asia with more than half of global ESCC cases actually occurred in China. Great geographical variation in incidence of ESCC implied a significant contribution of environmental and genetic factors to pathogenesis of this disease (Arnold, M. *et al.*, 2015; Katzka, D. A. and Metz, D. C., 2003).

1.2.2.1. Incidence

There were around 455,800 new esophageal cancer cases diagnosed globally every year, with approximately 398,800 cases of ESCC and 52,000 cases of EDAC (Arnold, M. *et al.*, 2015; Torre, L. A. *et al.*, 2015). ESCC is the predominant histological type of esophageal cancer in the world. Incidence of ESCC shows significant geographic variability worldwide. It occurs in remarkable high frequency in north of China, central and south of Asia, Japan, northern Iran, Turkey, east of Africa, which are known as the 'esophageal cancer belt' (Holmes, R. S. and Vaughan, T. L., 2007; Jobe, B. *et al.*, 2009; López, F. and Obiol, R. M., 2014; Stoner, G. D. and Gupta, A., 2001). Four regions in China were reported with a high incidence: Linxian of Henan province, Yang City of Shanxi, and Shenxian of Hebei as well as Nanao area of Guangdong province (Ke, L., 2002; Yang, C. S., 1980). In particular, Linxian with incidence rate exceeding 100 per 100,000 shows the highest incidence rate of esophageal cancer in the world (Yang, C. S., 1980). Whereas in low-risk areas like North America, the incidence rate is about 5.2 per 100,000 and generally remains stable. In Hong Kong, esophageal cancer was the eighteenth commonest cancer in Hong Kong, with 400 new cases in 2012 (Department of Health, HKSAR 2012).

1.2.2.2. Mortality

Esophageal cancer is an aggressive cancer with high mortality rate. In Hong Kong , esophageal cancer was identified as the ninth fetal cancer, leading to 329 deaths in 2013 for both sexes (Department of Health, HKSAR 2012). The overall 5-year survival rate was only around 20% (Tong, D. and Law, S., 2015).

A high mortality rate was also reported in China, with the incidence rate of 108.56 per 100,000, the mortality rate is 99.76 per 100,000(Yang, C. S., 1980). The most recent 5-year survival rate reported by Surveillance Epidemiology and End Results (SEER) registries (2005-2011) was 17.1% (National Cancer Institute, 2011). World Health Organization has predicted a rising trend for mortality caused by esophagus cancer in 2008. It was estimated that esophageal cancer will emerge as the fifteenth leading cause of death in 2030 from its position at twenty-fourth in 2004.

1.2.2.3. Risk Factors and Etiology

Incidence of ESCC showed great difference in geographic distribution worldwide and variation even occurred in different areas within a country. Such geographical clustering suggests a significant contribution of environmental and genetic factors to the pathogenesis of the disease(Katzka, D. A. and Metz, D. C., 2003;

McLaughlin, J. K. *et al.*, 2006). Chronic irritation and inflammation of esophageal mucosa have been postulated for the greatly increasing the incidence of ESCC. Hence, smoking, alcohol consumption, caustic damage to esophagus, ingestion of hot beverages and degraded foods constitute the major risk factors for ESCC. Dietary or life style associated with these risk factors probably increase the risk and is one of the reasons for the high incidence rate in some specfic populations or regions(Arnold, M. *et al.*, 2015; Enzinger, Peter C and Mayer, Robert J, 2003; Katzka, D. A. and Metz, D. C., 2003; Napier, K. J. *et al.*, 2014; Radojicic, J. *et al.*, 2012). With advanced recognition for the roles of oncogenes in pathogensis of ESCC, factors that cause activation of oncogene such as *protein tyrosine kinase 7* (Shin, W. S. *et al.*, 2013), *ras* (Ruol, A. *et al.*, 1990), *GAEC1*(Law, F. *et al.*, 2007) and *JK-1*(Tang, W. K. *et al.*, 2007)are regarded as etiologies for the disease.

1.2.2.4. Gender

Esophagus cancer is more common in male population than in female population. In 2012, the global incidence of ESCC was 7.7 per 100,000 for male population and 2.8 per 100,000 for female population. It is 3 to 4 times more common among male population than female world widely. For regions with high incidence rate, Eastern and South-East Asia got the incidence rate comparably higher for male

population with 13.6 per 100,000 for male and 4.3 for female(Arnold, M. *et al.*, 2015; Torre, L. A. *et al.*, 2015). According to statistics of Hong Kong, the male to female incidence ratio was approximately 4.6 to 1 (Department of Health, HKSAR 2012).

1.2.2.4.1. Alcohol & Tobacco

Alcohol consumption and smoking have been recognized as risk factors for many cancers. About 75% of patients with esophageal cancer adopted habits with alcohol and tobacco consumption(Pandeya, N. *et al.*, 2013). Increase in relative risk was identified with increasing volume of alcohol drinking(Organization, W. H., 2004). Substantial alcohol intake, especially combined tobacco consumption accounts for the carcinogenesis of esophageal cancer(Enzinger, Peter C and Mayer, Robert J, 2003). Many studies have reported a dose response relationship between the risk of esophageal cancer and the number of cigarettes smoked or smoking duration (Enzinger, Peter C and Mayer, Robert J, 2003; Katzka, D. A. and Metz, D. C., 2003; Tran, G. *et al.*, 2005).

1.2.2.4.2. Diet and Nutrients

High consumption of vegetable and fruits were found reducing risk of ESCC.

Vegetable and fruits are rich in antioxidant micronutrient such as ascorbate, carotenoids, vitamin E and selenium and bioactive compounds such as phenols, flavonoids and isoflavones which possess potent anti-carcinogenic properties (Lee, C. *et al.*, 2005; Tran, G. *et al.*, 2005).

Frequent consumption of extremely hot beverages has been associated with the development of ESCC. In South America, hot mate drinking double the risk of ESCC(De Stefani, E. *et al.*, 2014). While ingestion of beverages and food in high temperature contributed to nine-fold increase in risk of ESCC in Southern China(Lin, J. *et al.*, 2011).

1.2.2.4.3. Socioeconomic status

Income, education level, occupation and other variables indicate the life quality constitute socioeconomic status, was found to have association with the risk of ESCC (Holmes, R. S. and Vaughan, T. L., 2007). Population with higher levels of education was found to be at lower risk of ESCC in Taiwan(Lee, C. *et al.*, 2005). Similar inverse relationship was shown in the study conducted in Linxian that resident with highest education level and better living condition have the relative risk of 0.57 and 0.87 to that with lower SES. Low SES increases risk of EADC at well but the effect is not as strong as for ESCC(Holmes, R. S. and Vaughan, T. L., 2007).

1.2.2.4.4. Predisposition

Tylosis, a hereditary disorder characterized by focal hyperkeratosis and pigmentation of the palms and soles, carries 95% life time risk for development of ESCC.

Plummer-Vinson syndrome has also associated with increased risk for carcinogenesis of cervical esophagus(Katzka, D. A. and Metz, D. C., 2003; Mao, W.-M. *et al.*, 2011). Patients with esophageal achalasia are in comparably higher risk of developing ESCC with 33-fold increase over the general population(Meijssen, M. *et al.*, 1992).

1.2.3. Clinical presentation

Main symptom of esophageal cancer is difficulty in swallowing. It may get worse that feeling painfulness in swallowing solid food to liquid food at the later stage. Malnutrition, followed by significant weight loss is the typical symptom for esophageal cancer that it occurs in over 50% of patients. Some of the patients would experience symptoms likes vomiting, central chest discomfort behind the sternum as well as back discomfort between the shoulder blades(Department of Health, HKSAR 2012; Enzinger, P. C. and Mayer, R. J., 2003)

1.2.4. Diagnosis

The first step in diagnosis is the examination on patient's medical history including the onset of typical symptoms such as dysphagia and considerable weight loss, and evaluation of the patient's medical condition included alcohol and tobacco consumption. Then physician conducts appropriate diagnostic test for patients according to medical history and condition. As esophageal cancer usually spreads firstly to lymph nodes, physician checks for lymph nodes enlargement with finger and needle aspiration to remove cell tissue for microscopic evaluation. Besides, with biopsy cytological brushing is performed for accurate diagnosis.

A series of imaging tests including chest x-ray, endoscopy, double-contrast barium

swallow and computed tomography (CT) scan are used to diagnose esophageal cancer. Once esophageal cancer is diagnosed, the stage of the disease is determined (Katzka, D. A. and Metz, D. C., 2003).

1.2.5. Clinical Staging of esophageal cancer

After esophageal cancer is diagnosed, an accurate assessment of locoregional tumor, nodal staging and distant metastasis must be held for the treatment strategy. The prognosis is evaluated with the Tumor-node-metastasis (TNM) classification system for esophageal carcinoma of American Joint Commission on Cancer Staging (Table 1.2 & 1.3) (Jobe, B. *et al.*, 2009).

Table 1.2 American Joint Commission on cancer TNM classification for esophageal cancer

(Adopted from Katzka, D., and Metz, D, 2003)

Primary tumor (T)

T_X	Primary tumor cannot be assessed
T_0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T_1	Tumor invades lamina propria or submucosa
T_2	Tumor invades muscularis propria
T_3	Tumor invades adventitia
T_4	Tumor invades nearby structures

Regional lymph nodes(N)

N_X	Regional lymph nodes cannot be assessed
N_0	No regional lymph node metastasis
N_1	Regional lymph node metastasis

Distant metastasis (M)

M_X	Distant metastasis cannot be assessed
M_0	No distant metastasis
M_1	Distant metastasis

Tumors of the lower thoracic esophagus:

M1a	Metastasis in celiac lymph nodes
M1b	Other distant metastasis

Tumors of the midthoracic esophagus:

M1a	Not applicable
M1b	Nonregional lymph nodes and/or other distant metastasis

Tumors of the upper thoracic esophagus:

M1a	Metastasis in cervical nodes
M1b	Other distant metastasis

Table 1.3 American Joint Commission stage grouping

(Adopted from Katzka, D., and Metz, D, 2003)

Stage	Tumor	Node	Metastasis	Therapeutic options
Stage 0	Tis	N0	M0	Local ablative therapy
Stage I	T1	N0	M0	Surgery
Stage IIA	T2	N0	M0	Surgery
	T3	N0	M0	
Stage IIB	T1	N1	M0	Neoadjuvant therapy with or without surgery
	T2	N1	M0	
Stage III	T3	N1	M0	Neoadjuvant therapy with or without surgery
	T4	Any N	M0	
Stage IVA	Any T	Any N	M1a	Chemotherapy or radiation therapy with or without surgery
Stage IVB	Any T	Any N	M1b	Palliative treatment

1.2.6. Treatment for ESCC

1.2.6.1. Surgery

Resection is the best single-modality therapy for early stage patients. The surgical procedure is performed by determining the anatomic location of the tumor. In every surgery performed with curative intent, the primary goal is complete resection of tumor and involved lymph nodes. Surgery is an integral element in multi-modality therapy for regionally advanced cancers because patients who undergo neoadjuvant therapy have a 75% incidence of residual local disease amenable to resection. There is also a theoretical benefit of resection even for patient with pathologic response to neoadjuvant therapy. However, when cancer is found in advanced stage, complete resection is no longer suitable as a therapy strategy, surgical palliation may be considered instead (Katzka, D. A. and Metz, D. C., 2003).

1.2.6.2. Radiotherapy

Radiotherapy is applied as the single modality therapy in curative therapy for patients who are too ill and unable to tolerate either resection or combined chemoradiotherapy. Radiotherapy does not appear to offer a survival benefit, with survival rates at 5 years of less than 10%. The role of radiotherapy in the absence

of concomitant chemotherapy is limited. (Jobe, B. *et al.*, 2009; Katzka, D. A. and Metz, D. C., 2003)

1.2.6.3. Chemotherapy

Chemotherapy plays a central role in the treatment of esophageal cancer that one third of patients will have metastatic disease at the time of diagnosis. Chemotherapy as primary therapy using traditional cisplatin (CDDP) or 5-fluorouracil results in response rates up to 40%. Generally, squamous cell carcinoma is found to be more chemo-sensitive than adenocarcinoma. However, emergence of chemoresistance to chemotherapeutic drugs becomes the main obstacle in therapy. Therefore, development of novel effective chemotherapeutic compound is continuously conducted (Katzka, D. A. and Metz, D. C., 2003; Tong, D. and Law, S., 2015).

1.2.6.4. Chemo-radiotherapy

Chemo-radiotherapy is the combination of chemotherapy and radiotherapy, which is the most common treatment for esophageal cancer. The introduction of adjuvant chemo- and radiotherapy in recent years has improved the prognosis of esophageal cancer. However, the occurrence of radio-resistant cancer cells has always lowered

the effectiveness of Chemo-radiotherapy.(Jobe, B. *et al.*, 2009)

1.3. Oncogene *GAEC1*

GAEC1 (Gene Amplified in Esophageal Cancer 1) is an oncogene gene located at 7q22.1. It was first identified in ESCC because of the observation of frequent amplified 357bp sequence in inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR). mRNA of *GAEC1* contains 2052bp and encodes a nuclear protein of 109 amino acids(~15kDa) (Tang, J. C. *et al.*, 2001). It was characterized as a transforming oncogene as overexpression of *GAEC1* in NIH3T3 mouse fibroblasts enabled colony formation in soft agar, foci formation and undifferentiated sarcoma formation on nude mice after injection of *GAEC1*-transfected NIH 3T3 cells. *GAEC1* was found amplified and overexpressed in 34% of ESCC tumor and 60% of ESCC cell lines (Law, F. *et al.*, 2007). Besides, *GAEC1* amplification was associated with colorectal adenocarcinoma and significant difference was reported in cancer sub-sites and tumor types (Gopalan Chettiyar Padmam, V. *et al.*, 2013). However, no significant correlation was observed between *GAEC1* gene amplification and clinicopathological parameters and prognosis of ESCC in previous study of our group (Law, F. *et al.*, 2007).

1.4. Oncogene *JK-1*

JK-1, also known as FAM134B, is a gene located at chromosome 5p15.1, downstream to CTNND2 within the 5p contig (Figure 1.3). The sequence of *JK-1* has been published by National Center for Biotechnology Information (NCBI) in the category of "Human Genome Project". The full length of *JK-1* was 3184bp long which encoded 356 amino acid residues with molecular weight of 39.32 kD and pI of 4.39 (Compute pI/Mw tool).

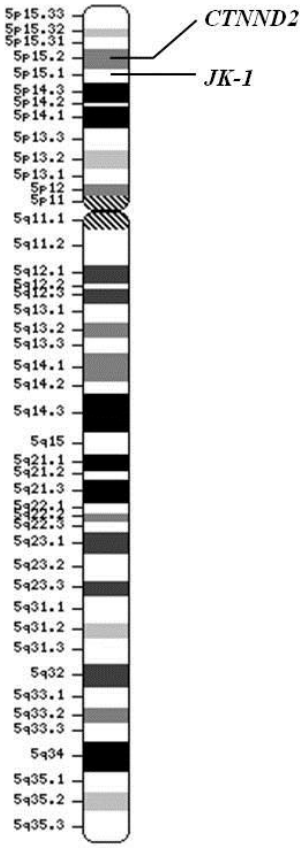


Figure 1.3 Diagram showing the position of *JK-1* on chromosome 5.
JK-1 is 3' downstream to CTNND2, The figure is not in scale. (Adopted from webpage of NCBI; http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?ORG=hum &MAPS=ideogr,est,loc&LINKS=ON&VERBOSE=ON&CHR=5)

JK-1 was previously demonstrated its tumor transforming properties *in vitro* and *in vivo* by the use of mouse fibroblast cells NIH 3T3 and human embryonic kidney HEK293 cells. NIH/3T3 cells and HEK293 overexpressing *JK-1* showed the increase in proliferation rate, acquired anchorage-independent growth ability, loss of contact inhibition and the formation of sarcoma after subcutaneous injection to athymic nude mice. Overexpression of *JK-1* was found frequently in ESCC cell lines and was associated with 30% of ESCC patient cases in multiplex RT-PCR analysis(Tang, W. K. *et al.,* 2007).

1.5. Anti-cancer Drug - Cisplatin

1.5.1. Molecular mechanism of anti-cancer actions of cisplatin

Cisplatin (CDDP), also named as cisplatinum or **c**is-**d**iammine**d**ichloro**p**latinum (II), is one of the most widely used chemotherapeutic drugs in the treatment of numerous human cancers for over three decades.

Cisplatin is a DNA-damage agent. It exerts its cytotoxicity to cancer cells via the formation of intra- and interstrand CDDP-DNA adducts, which can ultimately result in cell cycle arrest at G1, S, or G2-M and the induction of genetically programmed cell death (Dasari, S. and Tchounwou, P. B., 2014). It was also reported that CDDP could induce severe damages on normal organs because of its

general cytotoxic effects(Florea, A.-M. and Büsselberg, D., 2011).

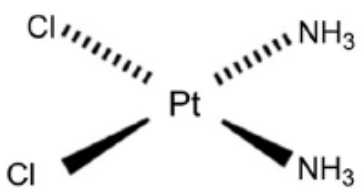


Figure 1.4 Structure of cisplatin

1.5.2. Molecular mechanism of cisplatin-resistance

Cisplatin is very effective for treatment of solid tumors. However, the development of resistance becomes one of the major problems with cisplatin-based treatment (Perez, R. P. *et al.*, 1993). *In vitro* studies on cell lines have shown that mechanisms of cisplatin-resistance are multi-factorial. These include decreased drug transport, increased cellular detoxification by cellular thiols glutathione (GSH) and metallothionein (MT), changes in DNA repair involving increased nucleotide excision repair and/or loss of mismatch repair, increased tolerance of DNA adducts, and defeat in the apoptotic cell death pathway (Galluzzi, L. *et al.*, 2012).

1.5.2.1. Membrane Transporters

Cisplatin can enter into cells by passive diffusion, facilitated diffusion and active transport. Na^+/K^+-ATPase transporter and solute carrier transporters were found

facilitating the entry of CDDP into the cells(Basu, A. and Krishnamurthy, S., 2010). Copper transporter 1 (CTR1) , a solute carrier transporter involved in copper homeostasis, was found to function as a cisplatin transporter, mediates cellular uptake of cisplatin (Ishida, S. *et al.*, 2002; More, S. S. *et al.*, 2010). A cell line with lower CTR1 expression were reported to have retarded uptaking of cisplatin(Song, I.-S. *et al.*, 2004).

However, CTR1 was not frequently under-expressed in cisplatin-resistant cell lines. Compared with respective parental cell lines, only one out of five small cell lung cancer cell lines acquiring resistance to cisplatin showed significant downregulation in expression level of CRT-1 (Song, I.-S. *et al.*, 2004). Cisplatin-resistant subline of ovarian carcinoma, osteosarcoma and cervix squamous cell carcinoma cell lines also had similar CRT1 expression level as their parental cell lines (Beretta, G. L. *et al.*, 2004; Yoshizawa, K. *et al.*, 2007).

As CTR-1 expression does not account for acquisition of cisplatin-resistance in majority, attention was attracted by two copper efflux P-type ATPases, ATP7A and ATP7B. Elevated expression of ATP7B was frequently reported in cisplatin-resistant cancer cells (Komatsu, M. *et al.*, 2000; Nakagawa, T. *et al.*, 2008; Yoshizawa, K. *et al.*, 2007). Cisplatin-resistant oral squamous cell carcinoma(OSCC) cells transfected with ATP7B siRNA becomes less resistant to

cisplatin (Yoshizawa, K. *et al.*, 2007).

ATP7B can serve as a marker for resistance to cisplatin that human ovarian cancer patients with higher ATP7B expression have relatively poor clinical outcome to cisplatin therapy(Nakayama, K. *et al.*, 2002).

One the other hand, some clinical studies showed that overexpression of ATP7A is associated with poor response to cisplatin treatment for patients with ovarian cancer (Samimi, G. *et al.*, 2003). Ovarian carcinoma cells transfected with ATP7A expressing construct acquired cisplatin-resistance (Samimi, G. *et al.*, 2004).

In addition, ATP7A and ATP7B are mainly localized in *trans*-Golgi network. However, it was found that they are distributed in more peripherally located vesicles in the cisplatin-resistant cells compared to the cisplatin sensitive cells in ovarian cancer. Changes in sub cellular localization of ATP7A and ATP7B thus may be associated to cisplatin-resistance (Kalayda, G. V. *et al.*, 2008).

Another group of researchers have examined association of ATP7A, ATP7B and Na^+/K^+-ATPase activity with the sensitivity to cisplatin in ovarian carcinoma cells. It was concluded that Na^+/K^+-ATPase is the major efflux transporter regulates intracellular CDDP accumulation in oral squamous carcinoma cells rather than ATP7A and ATP7B(Ahmed, Z. *et al.*, 2009).

It seems really difficult to correlate cisplatin-resistance with single, particular transporter because multiple transporters are involved in influx and efflux of cisplatin.

1.5.2.2. DNA repair system

Cisplatin induces apoptosis by causing DNA damage in cancer cells. Platinum atom of cisplatin binds covalently with purine bases at N^7 position, forming intra- and intercrosslinks. Formation of cisplatin-DNA adduct disrupts the structure of DNA, thus interferes cell division and results in apoptotic cancer cells death (Basu, A. and Krishnamurthy, S., 2010; Dasari, S. and Tchounwou, P. B., 2014).

This kind of DNA damage would be recognized by nucleotide excision repair (NER) system and/or mismatch repair (MMR) system. The relevance of DNA repair for cisplatin-resistance has been examined in many cancer types.

Enhanced cisplatin-DNA adduct removing ability was observed in cisplatin-resistant human ovarian cancer cells (Johnson, S. W. *et al.*, 1994a; Johnson, S. W. *et al.*, 1994b; Parker, R. *et al.*, 1991). Nucleotide excision repair (NER) is the main DNA repair pathway detecting and removing bulky helix-distorting DNA lesions induced by cisplatin. NER proficiency was observed in many kinds of cisplatin-resistant cancer cells.

Excision repair cross-complementation group 1 (ERCC1) is a protein playing critical role in NER. It dimerizes with xeroderma pigmentosum complementation group F (XPF), responsible for excision of the damaged DNA(Martin, L. P. *et al.*, 2008). Overexpression of ERCC1 has been correlated with relatively poor responsiveness to cisplatin treatment in ovarian cancer (Wang, L. *et al.*, 2008), bladder cancer (Bellmunt, J. *et al.*, 2007), hand and neck squamous cell carcinoma (Handra-Luca, A. *et al.*, 2007), cervical tumor (Britten, R. A. *et al.*, 2000) as well as non–small-cell lung cancer (NSCLC) (Olaussen, K. A. *et al.*, 2006). Moreover, knockdown of ERCC1 and XPF (xeroderma pigmentosum complementation group F) is able to enhance the response to cisplatin in NSCLC cells (Arora, S. *et al.*, 2010).

However, overexpression of ERCC1 was found both increase and decrease the sensitivity to cisplatin *in vitro*. Despite the fact that mechanism of acquiring cisplatin-resistance via ERCC1 under NER system is not fully understood, ERCC1 has been regarded as the biomarker in cisplatin-based therapy for cervical cancer and bladder cancer (Bellmunt, J. *et al.*, 2007; Britten, R. A. *et al.*, 2000)

MMR system is the major system recognizing single base mispairs or looped intermediates induced by cisplatin (Martin, L. P. *et al.*, 2008). Deficiency of MMR-related proteins, MSH2 and MLH1 were found associated to acquisition of

cisplatin-resistance in ovarian adenocarcinoma cells, endometrial cancer cells and colon cancer cells (Aebi, S. *et al.*, 1997).

It is believed that defect in MMR system results in failure of DNA adducts recognition. Cells with damaged DNA can escape from apoptosis and keep on proliferation, conferring resistance to cisplatin (Martin, L. P. *et al.*, 2008).

1.5.2.3. Interaction of cisplatin with cellular Thiols

Cisplatin is very reactive toward SH-containing molecules and thiols molecules. It has moderately high affinity with cysteines and methionines in cytoplasm. Therefore, these molecules may compete with nitrogen-containing DNA for binding and in turn detoxify cisplatin. Abundance of cysteines and methionines may confer cisplatin-resistance(Basu, A. and Krishnamurthy, S., 2010).

Elevated intracellular glutathione level has been closely correlated with cisplatin-resistance (Godwin, A. K. *et al.*, 1992; Ikeda, K. *et al.*, 2001; Okuno, S. *et al.*, 2003; Stewart, J. J. *et al.*, 2006). By using the rat pheochromocytoma cells, it has been proven that intracellular GSH level increases with the fold change of cisplatin-resistance in seven cisplatin-resistant sublines. Among seven cisplatin-resistant cell lines, the one with the highest intracellular GSH content was the most resistant one. Apply of L-buthionine-SR sulfoximine which reduced the level of

GSH, significantly reduced the resistance of the cells to cisplatin(Ikeda, K. *et al.*, 2001).

Glutathione S-transferase P1 (GSTP1), an enzyme that catalyzes the binding of cisplatin with GSH, was associated with cisplatin-resistance. A recent study has reported relatively higher expression of GSTP1 in osteosarcoma cisplatin-resistant variants compared with cisplatin-sensitive cell lines (Stewart, J. J. *et al.*, 2006).

On the other hand, numerous *in vitro* studies have shown that tumor cell lines with acquired resistance to cisplatin overexpress metallothionein (Kelley, S. L. *et al.*, 1988; Koropatnick, J. *et al.*, 1995; Sciavolino, P. *et al.*, 1992; Siegsmund, M. J. *et al.*, 1999). Metallothionein(MT) is a cysteine-rich protein, acts as a heavy metal binding protein, plays important role mainly in zinc and copper homeostasis as well as metal detoxification (Huska, D. *et al.*, 2009).

Immunocytochemistry analysis found that cisplatin-resistant ovarian cancer cells shows high level of MT in nuclei while no detectable MT level in nuclei of cisplatin-sensitive ovarian cancer cells. And cisplatin-resistant ovarian cells had increasing MT expression when they were exposed to increasing amount of cisplatin (Surowiak, P. *et al.*, 2007). A study using CH3 mice model demonstrated reversal of cisplatin- resistance in murine bladder tumor by administration of propargylglycine, an inhibitor to metallothionein synthesis (Saga, Y. *et al.*, 2004).

1.5.2.4. DNA damaging signaling

p53 acts as key regulator in cellular response to DNA damaging agent, likes cisplatin. It is stabilized and activated by phosphorylation upon DNA damage, triggers DNA repair, cycle arrest or apoptosis by trans-activating genes such as *p21*, *Bax*, *GADD45*, and *Mdm2* (Efeyan, A. and Serrano, M., 2007; McKay, B. C. *et al.*, 2001).

p53 status has been associated with sensitivity to cisplatin in cancer cells. Cisplatin-sensitive cancer cells frequently overexpress and own mutated p53. However, it was found that cisplatin-resistant sublines of head and neck cancer cell lines, have wild type p53 and reduced expression of p53 (Bauer, J. A. *et al.*, 2005; Bauer, J. A. *et al.*, 2007). It is possibly cells with mutated p53 were eliminated during cisplatin-selection. *In vitro* studies confirmed that p53 defect significantly lowers cisplatin-sensitivity in ovarian carcinoma cells (Branch, P. *et al.*, 2000; Fraser, M. *et al.*, 2008). Transduction of wild type p53 gene increased the sensitivity to cisplatin in ovarian cancer cells (Kigawa, J. *et al.*, 2001; Perego, P. *et al.*, 1996).

1.5.2.5. Apoptotic signaling

Compared with cisplatin-sensitive cancer cells, apoptotic proteins were found differently expressed in cisplatin-resistant cancer cells. This results in reduced susceptibility to cisplatin-induced apoptosis(Perego, P. *et al.*, 1996).

Mitogen-activated protein kinases (MAPKs) are involved in pathways to regulate cell proliferation, differentiation, and cell survival (Johnson, G. L. and Lapadat, R., 2002).

Three MARKs pathway have been characterized, including c-jun N terminal kinase (JNK) , p38 and extracellular signal-regulated kinase 1 and 2(ERK1/2).

Activation of MARKs has been shown to be critical for cisplatin-induced apoptosis. It is believed that MARKs are associated with acquisition of cisplatin-resistance (Brozovic, A. and Osmak, M., 2007; Wang, X. *et al.*, 2000).

Initial study found that mouse fibroblasts cells with Jun knockout were more resistant to cisplatin than normal cells while transfection of Jun-expression vector to Jun-lacking cells recovered the sensitivity to cisplatin (Sánchez-Pérez, I. and Perona, R., 1999). Decreased JNK activation was observed in cisplatin-resistant ovarian cancer cells (Cui, W. *et al.*, 2000). A study on ovarian cancer demonstrated that AKT2 activity promotes cisplatin-resistance via ASK1/JNK/p38 pathway (Hayakawa, J. *et al.*, 2000).

Inhibition of ERK1/2 activation was found increasing cisplatin-sensitivity in both

cisplatin-sensitive and cisplatin-resistant ovarian cell lines. It was proposed that ERK1/2 activation provides a cytoprotective effect against cisplatin in a series of cisplatin-resistant ovarian carcinoma cell lines. ERK1/2 was thus regarded as therapeutic approaches for the improving the efficacy of cisplatin treatment in the future (Cui, W. *et al.*, 2000; Hayakawa, J. *et al.*, 2000).

Protein kinase C (PCK), which acts upstream of the ERK signaling pathways, is essential in cisplatin-induced apoptosis (Clark, J. A. *et al.*, 2004; Lee, Y.-J. *et al.*, 2002). PCK activator, phorbol 12,13-dibutyrate, induced ERK1/2 phosphorylation, whereas PCK inhibitor bisindolymalemide resulted in reduced ERK1/2 activation in HeLa cells (Basu, A. and Tu, H., 2005). Decreased PKC activity was observed in cisplatin-resistant small cell lung carcinoma cell and ovarian cancer cells compared to their cisplatin-sensitive counterparts (Basu, A. and Weixel, K. M., 1995; Basu, A. *et al.*, 1996). In contrast, downregulation of PCK was found increasing sensitivity to cisplatin in osteosarcoma cell line and its cisplatin-resistant variant (Perego, P. *et al.*, 1993). The controversy may due to different roles by PCK isozymes in acquiring of cisplatin-resistance. A study showed that PKCα and PKCβ were downregulated in small cell lung cancer cells with cisplatin-resistance. Whereas PKCδ and PKCε were upregulated in cisplatin-resistant small cell lung cancer cells(Basu, A. *et al.*, 1996). It is believed that PKC

activity highly related to cisplatin-induced apoptosis but more evidence is required to confirm its association with acquisition of cisplatin-resistance.

Many studies have recognized Akt as one of the important factors in acquiring cisplatin-resistance in various cancers (Hamano, R. *et al.,* 2011; Liu, L.-Z. *et al.*, 2007; Peng, D.-J. *et al.*, 2010). It has been suggested that Akt promotes cisplatin-resistance by blocking mitochondrial action of p53 in the caspase-dependent mitochondrial apoptotic pathway. Inhibition of Akt sensitized cisplatin-resistant ovarian cancer cells by facilitating release of Smac (Yang, X. *et al.,* 2006). Akt is a member of serine/threonine kinase family which acts downstream of phosphoinositide 3-kinase(PI3K). PI3K/Akt pathway is essential for cell survival as it suppresses apoptosis. Enhanced Akt activity by unregulation of PI3K was found contributing to acquisition of cisplatin-resistance (Lee, S. *et al.*, 2005; Zhao, G. *et al.*, 2013).

1.6. Insulin-like growth factor (IGF) in drug resistance

Insulin-like growth factor system consists of six insulin-like growth factor-binding proteins (IGFBPs), insulin-like growth factors (IGFs), IGF-receptors (IGF-R) and IGFBP proteases (Firth, S. M. and Baxter, R. C., 2002; Kashyap, M. K., 2015). The bioavailability of IGF to IGF-R is regulated by IGFBPs, which bind to IGFs

with high affinity and only release IGF upon proteolysis by IGFBP proteases (Firth, S. M. and Baxter, R. C., 2002; Żesławski, W. *et al.*, 2001). Activation of IGF signaling pathway has demonstrated promoting tumor cells proliferation, differentiation, invasion, migration, survival and tumor expansion (Flier, J. S. *et al.*, 1997).

Upregulation of IGF-I (insulin like growth factor I) signaling, including elevated IGF-I, overexpression of IGF-IR and decreased expression of IGFBPs, were frequently detected in ESCC (Li, F. *et al.*, 2010; Ma, W. *et al.*, 2014) and other cancers (Jones, R. *et al.*, 2009; Mitsiades, C. S. *et al.*, 2002; Surmacz, E., 2000). Recent findings revealed that hyperactivation of IGF signaling was associated with reduced sensitivity to cisplatin-based chemotherapy in several types of cancers including ESCC (Ma, W. *et al.*, 2014),ovarian cancer (Eckstein, N. *et al.*, 2009), lung cancer (Sun, Y. *et al.*, 2012) and mesothelioma (Kai, K. *et al.*, 2009).

Inhibition of IGF signaling by IGFBP3, has been shown to lead to increased sensitivity to cisplatin in cisplatin-resistant lung cancer cells (Sun, Y. *et al.*, 2012). Studies on the acquisition of cisplatin-resistance mediated by other members of IGFBPs are scanty.

1.7. Quinoline compounds

Quinoline derivatives have been widely reported to possess a broad range of pharmaceutical activities and can be isolated from different plant sources (Chan, S. H. *et al.*, 2013). The first commonly known natural quinoline compound, 4-hydroxy-6-methoxy-quinoline-2-carboxylic acid (Figure 1.5), was extracted from *Ephedra pachyclada ssp. Sinaica* which has been widely used in traditional herbal medicine against allergy, inflammation, microbial and cardiovascular diseases, and cancer (Kumar, S. *et al.*, 2009; Michael, J. P., 1998).

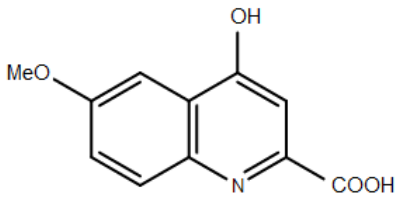


Figure 1.5 Structure of 4-hydroxy-6-methoxyquinoline-2-carboxylic acid.

8-hydroxyquinoline and its derivatives were also reported as natural products such as those isolated from the roots of *C. diffusa* with phytotoxic activities (Tharayil, N. *et al.*, 2009) and *S. corniculata* with antibacterial and antifungal activities (Jeon, J. H. *et al.*, 2009). These facts inspired researchers to further improve the efficacy and potency of quinoline by modifying its structure. Our previous findings also revealed that the 8-hydroxyquinoline derivatives showed relatively promising *in*

vitro and *in vivo* anti-cancer effects (Chan, S. H. *et al.*, 2013; Lam, K. H. *et al.*, 2014a), and anti-bacterial effects (Lam, K. H. *et al.*, 2014b) implying the importance of the 8-hydroxyl group for their biological actions.

A series of novel 2-substituted 8-hydroxyl quinoline derivatives were synthesized by our research group, an example used in the present study is 91b1 which was selected based on the structural-activity study in the previous PhD study of Dr. Penny Chan Sau-hing from our research group:

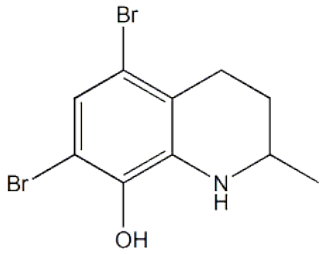


Figure 1.6 Structure of 91b1

In this study, attempt was further made to investigate the novel quinoline derivative 91b1, 5,7-dibromo-1,2,3,4-tetrahydro-2-methylquinolin-8-ol which was based on the core structure of the 8-hydroxyquinoline derivatives that is also available from natural sources as reported by our group before (Chan, A. S. C. *et al.*, 2009; Chan, S. H. *et al.*, 2013; Lam, K. H. *et al.*, 2013), and to examine its *in vitro* anti-cancer effects on ESCC cells.

Chapter 2 Aims and Objectives

The present study has four aims:

(I) To study the effect of suppressing the oncogene *GAEC1* and identify the downstream regulated genes of *GAEC1* in ESCC and the clinicopathological significance.

(II) To correlate the overexpression of *JK-1* with clinicopathological parameters of ESCC patients and study the effect of suppressing *JK-1* expression.

(III) To characterize the gene expression profile for cisplatin-resistant ESCC cells and to reverse cisplatin-resistance.

(IV) To investigate the anti-cancer effect of 91b1 on ESCC cell lines and characterize the gene expression profile after treatment

The objectives related to Aim (I) are listed as follows:

i. To evaluate the effect of knocking down the expression of *GAEC1* by RNA interference;

ii. To identify the downstream candidate genes regulated by the suppressed *GAEC1* expression;

iii. To correlate the downstream-regulated gene of *GAEC1* with ESCC patients' clinicopathological features.

The objectives related to Aim (II) are listed as follows:

i. To knockdown the overexpression of the oncogene *JK-1* in ESCC cells using RNA interference;

ii. To study the reversal of tumor transforming properties with *JK-1* knockdown in ESCC cells which have *JK-1* overexpression;

iii. To correlate the overexpression of JK-1 with ESCC patients' clinicopathological features to predict the prognostic significance of JK-1.

The objectives related to Aim (III) are listed as follows:

i. To identify the gene expression profile which is induced by cisplatin-resistance in ESCC cells;

ii. To reverse the drug resistance phenotypes by targeting the most affected candidate gene.

The objectives related to Aim (IV) are listed as follows:

i. To determine the dose-dependent cytotoxicity effects of 91b1on ESCC cells;

ii. To identify the changes in gene expression profile which is induced by the cytotoxic effects of 91b1;

iii. To study the most affected candidate gene after 91b1 treatment for its mechanisms in tumor growth in ESCC.

Chapter 3 Materials and Methods

3.1. Cell lines and cell culture

3.1.1. ESCC cell lines

ESCC cell lines of Japanese origin KYSE150, KYSE450, KYSE30, KYSE70 and KYSE510 were purchased from DSMZ (Braunschweig, Germany).

ESCC cell lines of Hong Kong origin SLMT-1(Tang, J. C. O. *et al.*, 2001) and HKESC-4 (Cheung, L. C. *et al.*, 2007) were kindly provided by Professor Gopesh Srivastava of the Department of Pathology, The University of Hong Kong.

KYSE150, KYSE450 and KYSE30 were maintained in 45% RPMI 1640 medium (Gibco,USA), 45% Ham's F12 (Gibco,USA) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100μg/mL penicillin (Gibco,USA) and 100μg /mL streptomycin (Gibco,USA) (Shimada, Y. *et al.*, 1992).

KYSE70 and KYSE510 were maintained in RPMI 1640 medium (Gibco,USA) supplemented with 10% FBS, 100μg/mL penicillin and 100μg /mL streptomycin (Shimada, Y. *et al.*, 1992).

SLMT-1 and HKESC-4 cells were maintained in minimum essential medium alpha (MEMα, Gibco, USA) supplemented with 20% FBS, 100 μg/mL penicillin and 100 unit/mL streptomycin (Cheung, L. C. *et al.*, 2007; Tang, J. C. O. *et al.*, 2001).

Cells were maintained at 37℃ in a humidified incubator with 5% CO_2. Trypsinization was performed when the density of cells reached 80% confluence. The cells were washed by phosphate buffered saline (PBS) and split in 1:3 ratio by adding 0.05% trypsin in 0.05% EDTA (Gibco,USA) for sub-culturing.

3.1.2. Immortalized non-tumor esophageal epithelial cell lines

Non-tumor esophageal epithelial cell lines NE-1 (Deng, W. *et al.*, 2004) and NE-3 (Zhang, H. *et al.*, 2006) (immortalized by the induction of genes E6 E7 of human papillomavirus type 18) were kindly provided by Professor George S.W. Tsao of the Department of Anatomy, The University of Hong Kong. The cells were cultured in keratinocyte serum-free medium (KSFM, Gibco,USA) without any serum and with 100μg/mL penicillin and 100μg/mL streptomycin. The cells were maintained at 37℃ in a humidified incubator with 5% CO_2. Trypsinization was performed as previously described. A splitting ratio of 1:2 was used for sub-culturing.

3.1.3. Non-tumor embryonic kidney cells

HEK293, embryonic kidney cells, was purchased from the American Type Culture Collection (ATCC) (Graham, F. *et al.*, 1977) and used as non-tumor cells for MTS cytotoxicity assay of 91b1. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% FBS, 100μg/mL penicillin and 100μg /mL streptomycin at 37℃ in a humidified incubator with 5% CO_2. Trypsinization was performed as previously described in section 3.1.1 when the density of cells reached 80% confluence. A splitting ratio of 1:5 was used for sub-culturing.

3.1.4. Transfected KYSE150 cell lines

For the study of *GAEC1*, KYSE150-pSilencer-P3-4 cells, KYSE150-pSilencer-ve and KYSE150-pcDNA3.1-GAEC1 cells were established from KYSE150 cells transfected with *GAEC-1* targeted siRNA expression vector (pSilencer-P3-4), pSilence2.1-U6 Neo negative control (pSilencer-negative control vector) and GAEC-1 expression vector (pcDNA3.1-GAEC1). The detailed procedures for establishment of these three cell lines were described in section 3.7.1. KYSE150-pSilencer-P3-4 cells, KYSE150-pSilencer-ve cells and KYSE150-pcDNA3.1-GAEC1 cells were maintained in 45% RPMI 1640 medium, 45% Ham's F12

supplemented with 10% FBS, 100μg/mL penicillin, 100μg /mL streptomycin and 400μg/mL geneticin (G418, Gibco, USA) at 37℃ in a humidified incubator with 5% CO_2. Trypsinization was performed as previously described in section 3.1.1 when the density of cells reached 80% confluence. A splitting ratio of 1:5 was used for sub-culturing.

3.1.5. Transfected KYSE70 cell lines

For study of *JK-1*, KYSE70-sh1 cells and KYSE70-shcon cells were established from KYSE70 cells transfected with *JK-1* siRNA expression vector (*JK-1*-siRNA-psiNU6) and mock vector (control-siRNA-psi-nU6) respectively. The detailed procedures for establishment of these two cell lines were described in section 3.7.1. KYSE70-sh1 cells and KYSE70-shcon cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100μg/mL penicillin ,100μg /mL streptomycin and 1μg/mL puromycin (Gibco, China) at 37℃ in a humidified incubator with 5% CO_2. Trypsinization was performed as previously described in section 3.1.1 when the density of cells reached 80% confluence. A splitting ratio of 1:5 was used for sub-culturing.

3.1.6. ESCC cell line with cisplatin-resistance

A cisplatin-resistant cell line, SLMT-1/CDDP1R was established from cell line SLMT-1 by culturing in an increasing concentration of cisplatin (CDDP, Sigma-Aldrich, USA). The starting concentration of cisplatin used was 0.1μg/mL. Proliferation cells were repeatedly subcultured in medium containing an increasing concentration of cisplatin from 0.1μg/mL, 0.2μg/mL,0.5μg/mL, 0.75μg/mL to 1.00μg/mL.

SLMT-1/CDDP1R was maintained in culture medium of SLMT-1 (refer to section 3.1.1) with 1.00μg/mL of cisplatin at 37℃ in a humidified incubator with 5% CO_2. Trypsinization was performed as previously described in section 3.1.1 when the cells reached 80% confluence. A splitting ratio of 1:3 was used for sub-culturing.

3.1.7. Transfected SLMT-1 and cisplatin-resistant SLMT-1 cell lines

Cell lines SLMT-1-IGFBP5 and SLMT-1-pcMV3 were established by SLMT-1 cells transfected with *IGFBP5* expressing vector and pcMV3 mock vector. Similarly, cell lines SLMT-1-R-IGFBP5 and SLMT-1-R-pcMV3 were established by SLMT-1/CDDP1R cells transfected with IGFBP-5 expressing vector and pcMV3 mock vector. Details procedures for transfection were described in the

following section (section 3.7.1). SLMT-1-IGFBP5, SLMT-1-pcMV3 and SLMT-1-R-IGFBP5 were maintained in MEMα supplemented with 20% FBS, 100 μg/mL penicillin, 100 unit/mL streptomycin, 400ug/mL Hygromycin B (Invitrogen, USA).

SLMT-1-R-pcMV3 transfected cells were maintained in MEMα supplemented with 20% FBS, 100 μg/mL penicillin,100 unit/mL streptomycin, 1.00μg/mL of cisplatin, 400ug/mL Hygromycin B (Invitrogen, USA)

All the cells were maintained at 37℃ in a humidified incubator with 5% CO_2. Trypsinization was performed as described in section 3.1.1.1 when the cells reached 80% confluence. A splitting ratio of 1:3 was used for sub-culturing.

3.2. ESCC patient specimens

For the study of *GAEC1*, a tissue microarray (TMA) containing 132 archived ESCC paired non-tumor and tumor fresh tissue samples were prepared after esophagectomy with patients' consent at the Department of Surgery, Queen Mary Hospital, Hong Kong from 2001 to 2006. All the specimens were collected from patients who have received no prior treatment directed to the primary ESCC. 101 ESCC patient specimens were acquired with clinical and histological information reported by Dr. K. W. Chan from the Department of Pathology, The University of

Hong Kong. While an addition of 31 ESCC patient specimens were acquired with only age and gender. Table 3.1 summarizes the information of the cases.

Table 3.1 Summary of clinicopathological characteristics of patients with ESCC

Characteristic	No. of Patients
Age, years	
Mean	65.41
Standard deviation	10.25
Range	41-87
Sex	
Male	102
Female	30
TNM stage	
0/I/II	18
III/IV	83
Tumor depth (Depth of invasion)	
T1-T3	79
T4	22
Lymph node metastasis	
N0	32
N1	69
Distant metastasis	
M0	66
M1	35
Differentiation	
Well	16
Moderate	59
Poor	26

For the studies of *JK-1* and 91b1, paraffin embedded tissues containing 26 tumor tissues, 15 non-tumor tissues and 16 pre-malignant tissues were also collected after esophagectomy with patients' consent at the Department of Surgery, Queen Mary Hospital, Hong Kong from 1990 to 2001. All the specimens were collected from patients who have received no prior treatment directed to the primary ESCC. The clinical and histological information of ESCC patient specimens were reported by specialist pathologists of the Department of Pathology, Queen Mary Hospital, Hong Kong. The clinicopathological information was summarized in Table 3.2.

Table 3.2 Summary of clinicopathological characteristics of patients with ESCC

ESCC patient no.	Age	Sex	Differentiation	TMN	overall stage	Survival (months)
1	60	M	M	T3N1M0	III	6.82
2	74	M	M	T3N1M0	III	20.79
3	77	M	W	T4N1M0	III	2.59
4	70	M	M	T3N0M1	IVB	2.75
5	76	F	M	T3N0M0	IIA	8.79
6	70	M	M	T3N1M0	III	33.34
7	72	F	M	T3N1M0	III	2.1
8	47	M	M	T3N1M0	III	1.28
9	58	M	M	T3N0M0	IIA	3.84
10	66	F	P	T3N0M0	IIA	28.59
11	72	M	M	T3N0M0	IIA	5.44
12	46	M	W	T3N1M0	III	1.64
13	53	F	M	T3N0M0	IIA	14.07
14	71	F	W	T2N1M0	IIB	14.43
15	69	M	M	T3N0M0	IIA	4.75
16	69	M	M	T3N1M0	III	10.23
17	57	F	W	T4N0M0	III	3.08
18	45	M	M	T4N1M0	III	6.39
19	77	F	W	T3N1M0	III	85.64
20	52	M	M	T4N0M0	III	8.43
21	46	F	M	T4N1M0	III	43.15
22	73	M	M	T3N1M0	III	33.67
23	66	F	W	T3N0M0	IIA	28.59
24	57	M	W	T4N1M0	III	9.57
25	47	M	W	T4N1M0	III	11.21
26	66	M	W	T3N0M1	IVA	152.56

3.3. Quinoline compound 91b1

The novel 2-substituted 8-hydroxyl quinoline derivative, 91b1 (5,7-dibromo-1,2,3,4-tetrahydro-2-methylquinolin-8-ol) was kindly synthesized by Dr. Penny Chan Sau-hing from our group the and it was used in the present study to examine its *in vitro* anti-cancer effects on esophageal cancer. The structure of 91b1 has been confirmed by NMR study.

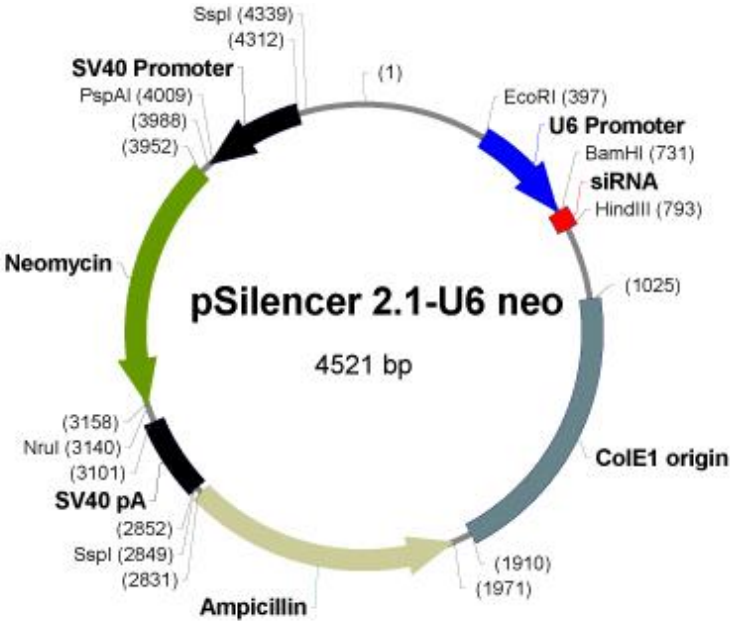
3.4. Preparation of small interfering RNA (siRNA) expression vector

For the study of *GAEC1*, pSilencer 2.1-U6 neo siRNA expression vector (Ambion, USA) was applied to express specific small-hairpin siRNAs (shRNAs) targeting *GAEC1*. 63nt oligonucleotides encoding the specific siRNAs were purchased from Integrated Device Technology and the insert sequences(P3-4) are

Top strand:
5'-
GATCCGAAGTGGCTTCTGGATTAATTCAAGAGATTAATCCAGAAGC
CACTTCTTTTTTGGAAA-3'
Bottom strand:
5'-
AGCTTTTCCAAAAAAGAAGTGGCTTCTGGATTAATCTCTTGAATTA
ATCCAGAAGCCACTTCG-3'.

These oligonucleotides were annealed and ligated to the BamHI and HindIII sites of the pSilence2.1-U6 Neo siRNA expression vector to obtain the expression

plasmids pSilencer-P3-4. The inserted sequences were confirmed by DNA sequencing. The pSilence2.1-U6 Neo negative control (pSilencer-negative control vector) was a negative control plasmid that encoded a small-hairpin siRNA which sequence had limited homology to known sequences in the human, mouse and rat genome (Zhang, W. *et al.*, 2009).



U6 Promoter: 397-731
SV40 early Promoter: 3988-4312
Neomycin: 3158-3952
SV40 early pA signal: 2852-3101
Ampicillin: 1971-2831
ColE1 origin: 1025-1910

Figure 3.1 A map of pSilencer 2.1-U6 neo vector
(Adopted from instruction manual of pSilencer^{TM} neo, Ambion)

3.5. Transformation of competent cells

For the study of *GAEC1*, about 20 μL of the ligation product of siRNA expression vector (pSilencer-P3-4) and pSilencer-negative control vector were transformed

into 200 μL of One Shot® OmniMax™ 2-T1 chemically competent E.coil cells respectively (Invitrogen, USA). The cells were then chilled on ice for 15 min, followed by heat shocked in 42°C water bath for 90 sec and incubation on ice for 2 min. About 800 μL of LB broth was added to the cells and the culture was incubated at 37°C for 1.5 hours. Then, about 200 μL of transformation culture was plated onto duplicate agar plates containing 100 μg/mL ampicillin. The plates were incubated for about 18 hours at 37°C for standard ampicillin selection. After the transformation, the single colonies were picked and grown in 5 mL LB broth containing 100 μg/mL ampicillin and incubated at 37°C for 18 hours. Glycerol stock was prepared with 50% glycerol. The glycerol stock was stored in -80°C for later application.

3.6. Plasmid extraction

For study of *GAEC1*, the plasmids of pSilencer-P3-4 and pSilencer-negative control vector were isolated from the transformed cells by Hybrid-QTM Plasmid Total DNA Purification Kit (GeneAll, Korea). Clones of pSilencer-P3-4 and pSilencer-negative control vector were inoculated in 5mL medium containing 100 μg/mL ampicillin. The cultures were then grown at 37℃ for 18 hours with shaking at 250 rpm. 3mL of culture was pelleted by centrifugation for one minute at

13000rpm. Supernatant was discarded and the cell pellet was resuspended in 170μL cell suspension buffer. 170μL lysis buffer was then added to the cell suspension and mixed by inverting the tube gently for 4 times. The cells were allowed to lyse for 1 minute. After the cell lysis, 250μL neutralization buffer was added and mixed immediately by inverting the tube gently for 5 times. All of the lysate was transferred to EzClear™ Column stack and centrifuged at 13000rmp for 1 minute. The upper EzClear™ Filter Column unit and the flow-through fraction were discarded. 500μL wash buffer AW was applied to the spin column for removal of residual protein. The column was centrifuged for 1 minute and flow-through fraction was discarded. Another washing buffer, buffer PW was applied to remove salts and other cellular components. 700μL of buffer PW was added to the spin column and centrifuged for 1 minute, the through-flow was removed. The spin column was centrifuged for one extra minute to remove residual washing buffer. The spin column was transferred to a new 1.5mL eppendorf tube. 90μL of sterile water was added and inoculated at room temperature for one minute. The spin column and eppendorf were centrifuged at 13000rpm for 1 minute for the elution of plasmid DNA. The plasmid DNA was used immediately or stored at -20 ℃.

3.7. Transfection

3.7.1. Transfection of expression vector

(1) For study of *GAEC1*, pSilencer-P3-4 was transfected into KYSE150 cells for suppressing *GAEC1* expression. And pSilencer-negative control vector was also transfected into the cells as control. In addition, pcDNA3.1-*GAEC1* vector (Law, F. *et al.*, 2007) was transfected into KYSE150 cells for cDNA microarray analysis. Map of pcDNA3.1(-) vector showing the restriction site of cloning was shown in Figure 3.2.

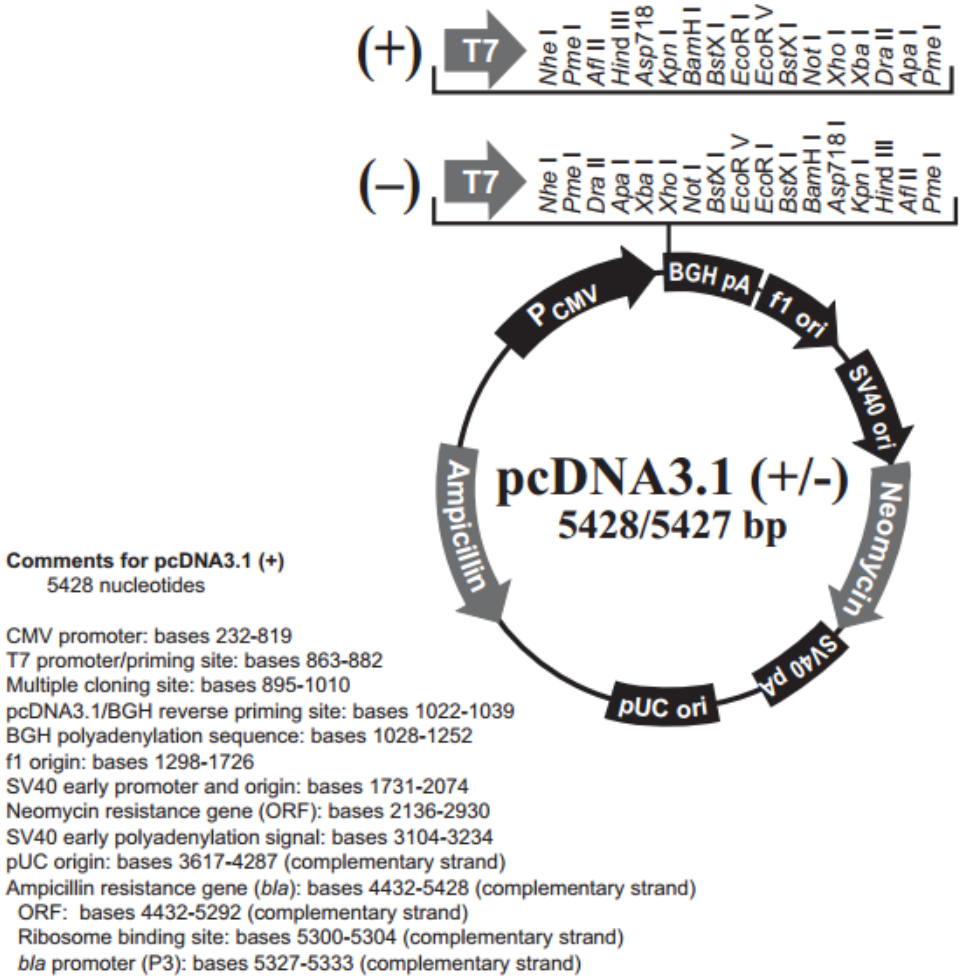


Figure 3.2 A map of pcDNA3.1(-)and pcDNA3.1(+) vectors
(Adopted from user manual of pcDNA™3.1(+) pcDNA™3.1(–), Invitrogen)

(2) For study of *JK-1*, siRNA expression vector *(JK-1*- siRNA-psiNU6) specific for *JK-1* interference was purchased from GeneCopoeia (GeneCopoeia, Rockville, USA). Short hairpin RNA (shRNA) sequence 5'-GATCCGGAACAGTGACCAAACCTTTTCAAGAGAAAGGTT TGGT CACTGTTCTTTTTTGGAATT-3' was cloned by the manufacturer under the control of the U6 promoter into the psi-nU6 vector (Figure 3.3). *JK-1*-siRNA-

psiNU6 was transfected into KYSE70 cells for suppressing *JK-1* expression. And control-siRNA-psi-nU6 vector was also transfected into the cells as control.

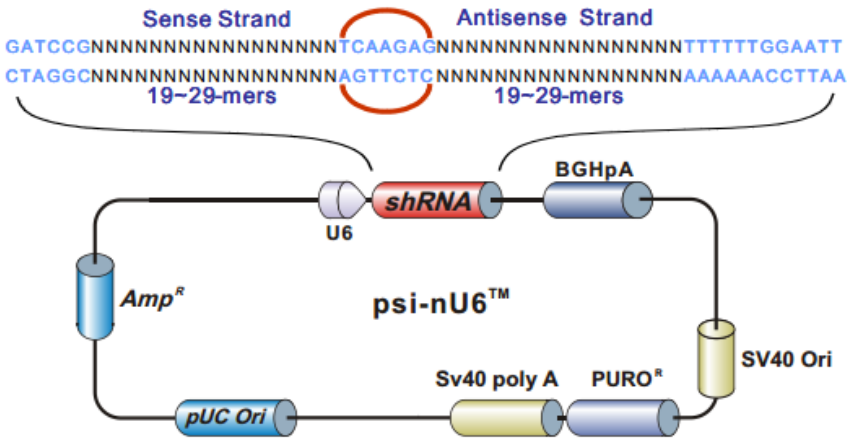


Figure 3.3 A map of psi-nU6 vector
(Adopted from datasheet of OmicsLinkTM shRNA Expression, GeneCopoeia)

(3) For study of CDDP-resistance, *IGFBP5* expressing vector *IGFBP5*/pcMV3-C-Myc (Figure 3.4, Sino Biological Inc.) and Myc tagged pcMV/hydro-negative control vector(Figure 3.5, Sino Biological Inc.) were transfected into parental SLMT-1 or SLMT-1/CDDP1R cells for examining the reversal of cisplatin-resistance.

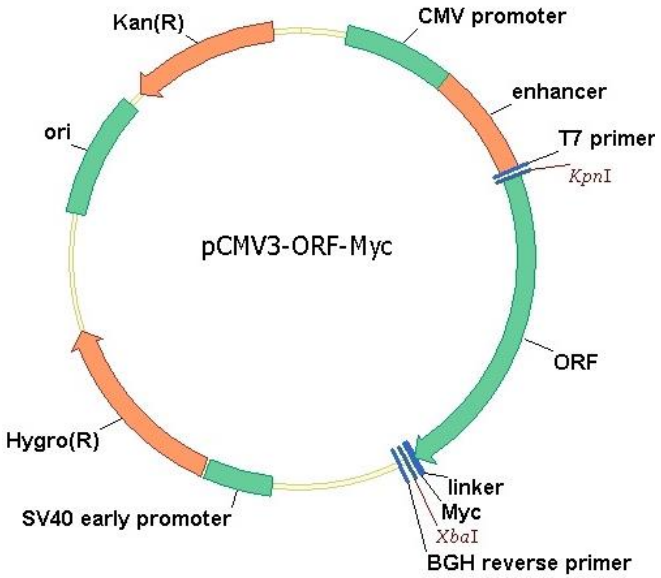


Figure 3.4 Physical map of IGFBP5-expression vector (IGFBP5/pcMV3-C-Myc)

(Adopted from Datasheet of Human IGFBP5 ORF mammalian expression plasmid, C-Myc tag)

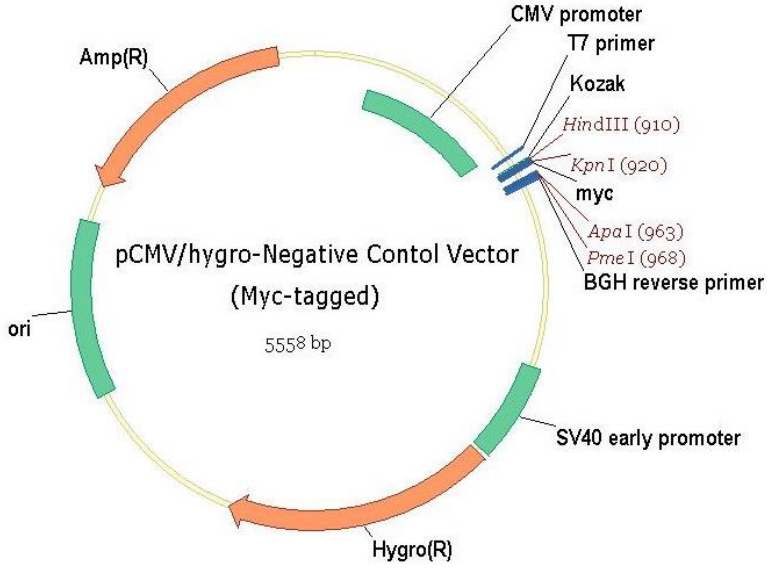


Figure 3.5 Physical map of pcMV/hygro-negative control vector (Myc-tagged)

(Adopted from Datasheet of pcMV/hygro-Negative Control Vector ,Myc-tagged)

(1) KYSE150 cells; (2) KYSE70 cells; (3) SLMT-1 cells or SLMT-1/CDDP1R cells were seeded onto a 6-well plate (Nunc, Danmark) at density of $2x10^5$ cells in 2mL culture medium and incubated at 37℃ with 5% CO_2 overnight. For each well,

2μg of (1) pSilencer-P3-4, pSilencer-negative control vector or pcDNA3.1-GAEC1 vector; (2) JK-1- siRNA-psiNU6 or control-siRNA-psi-nU6;

(3) IGFBP5/pcMV3-C-Myc or pcMV/hydro-negative control vector plasmid DNA was added to pre-warmed serum-free RPMI medium to give 100μL of DNA-medium mixture. 3μL of FuGene®HD transfection reagent (Promega,USA) was mixed with DNA containing medium and incubated at room temperature for 15 mins. The cells were washed with PBS. To each wells, 1.9mL culture medium was added. Transfection mixture was added to the wells and mixed gently after 15 mins of incubation. The cells were incubated with transfection mixture medium for 48hours. After 48-hour transfection, culture medium containing (1) 50-1,000 μg/mL geneticin (G418,Gibco,USA) (2) 0.5μg/mL-1μg/mL puromycin (3) 50-400μg/mL hygromycin was applied for selection of transfected cells. The colonies were expanded to stable lines

(1) KYSE150-pSilencer-P3-4, KYSE150-pSilencer-ve and KYSE150-pcDNA3.1-GAEC1; (2) KYSE70-sh1 and KYSE70-shcon; (3) SLMT-1-IGFBP5, SLMT-1-pcMV3, SLMT-1-R-IGFBP5 and SLMT-1-R-pcMV3 cell lines in selection medium after two months. Transfected cells were harvested by trypsinization once they grew stably and subjected to RNA extraction and PCR analysis to examine the expression of *GAEC1, JK-1 and IGFBP5*. The culture

medium of each cell lines was described in section 3.1

3.7.2. Transfection of siRNA

siRNA targeting *IGFBP5* (Ambion) was transfected into SLMT-1 cells by Lipofectamine RNAiMax Reagent (Invitrogen). The siRNA sequences were:

Sense: 5'-GCAAGUCAAGAUCGAGAGATT-3'
Antisense: 5'-UCUCUCGAUCUUGACUUGCTC-3'

SLMT-1 cells were harvested by trypsinization as described in section 3.1. The cell number was counted by hemocytometer under microscope. Cells were seeded into a flat-bottom 96-well plate (SPL, Korea) at density of 2×10^3 cells per well and maintained in 100μL of its culture medium overnight. Transfection mixture was prepared with 0.3mM of siRNA and 3%v/v of lipofectamine reagent in 10μL of Opti-MEN Reduced Serum Medium (Gibco, USA). The transfection mixture was then incubated in room temperature for 5mins. Medium of cells were replaced with culture medium in 90μL per well and 10μL of mixture was added to each well. The cells were subjected to qPCR analysis with procedures described in section 3.8.3.2 and MTS cytotoxicity assay (section 3.11) after incubated with the transfection medium for 48 hours.

3.8. Gene expression studies

For study of *GAEC1*, the expression of *GAEC1 in* KYSE 150 parental cells was compared with NE-1 and KYSE150-pSilencer-P3-4 cells.

For study of *JK-1*, gene expression of *JK-1* in KYSE70, KYSE150, KYSE510 and SLMT-1 were first compared with NE-3. The expression of *JK-1* in KYSE70-sh1 and KYSE70-shcon were then compared with KYSE70 after transfection of JK-1-siRNA-psiNU6 vector and control-siRNA-psi-nU6 vector.

For study of CDDP-resistance, gene expression of *IGFBP5* in SLMT-1, SLMT-1/CDDP1R, SLMT-1-IGFBP5, SLMT-1-pcMV3, SLMT-1-R-IGFBP5 and SLMT-1-R-pcMV3 were examined.

For study of 91b1, expression of *CCL5* in HKESC-4, KYSE450, KYSE510, KYSE150, KYSE30, HEK293 and NE-3 were first examined. The expression of *CCL5* in 91b1-treated (6.5μg/mL, 9.5μg/mL and 21μg/mL for 48hours) KYSE150, KYSE450, KYSE510 and HKESC-4 cells were compared with respective DMSO-treated cells for evaluating the effect of 91b1 treatment. All the gene expression studies were conducted with the following procedures (Section 3.8.1-3.8.3).

3.8.1. Total RNA extraction

About 1×10^7 of all tested cells were harvested by cell scrapper (SPL, Korea) for total RNA extraction using RNeasy Mini Kit (Qiagen,USA). Cells were first disrupted and homogenized by adding 350μL Buffer RLT. 350μL of 70% ethanol was then added to the lysate, and mixed well by pipetting. The samples were then transferred to the RNeasy Mini spin column and centrifuged for 30secs at 13,000 rpm. All the flow-through was discarded. Total RNA was trapped by the membrane. 700μL Buffer RW1 was added to the spin column and centrifuged to remove cell debris. 500μL of Buffer RPE was applied to the spin column and centrifuged for 1 min twice. 50μL RNase-free water was added and centrifuged for 1 min at 13,000 rpm to elute the total RNA. The RNA was used immediately or stored at -80℃ until used.

3.8.2. cDNA synthesis

cDNA stands were synthesized from total RNA using the GoScript™ Reverse Transcription System (Promega, USA). 2μg of RNA was incubated with reaction mixture (1×PCR buffer, 2.5mM $MgCl_2$, 0.5mM dNTP, 2.5μM random hexamer, ribonuclease inhibitor (1U/μL) and MuLv reverse transcriptase (2.5U/μL) at room temperature for 5mins. The reverse transcription reaction was performed at 42℃

for 1 hour followed by 70℃ for 15mins. The cDNA was used immediately or stored at -20℃ until used.

3.8.3. Polymerase chain reaction (PCR) analysis

3.8.3.1. Multiplex PCR

For study of *GAEC1*, the expression level of *GAEC1* in the parental cell lines and transfected cells was analyzed by RT-PCR analysis. cDNA (~2 μg) produced by reverse transcription from the RNA of KYSE150, KYSE150-pSilencer-P3-4, KYSE150-pSilencer-ve and NE-1 cells, were amplified after initiation at 95℃ for 5 mins, 35 cycles of denaturation at 95℃ for 1 min and annealing at 50℃ for 1 min and extension at 72℃ for 1 min, followed by a final extension at 72℃ for 6 mins, by using specific PCR primer pair for the *GAEC1* and the specific *β-Actin* gene primers acting as an internal control for normalizing the cDNA quantity. Primers for *GAEC1* and *β-Actin* are shown as following:

β-Actin	
β-Actin-F	5'-GTGGGGCGCCCCAGGCACCA-3'
β-Actin-R	5'-CTCCTTAATGTCACGC ACGATTTC-3'
GAEC1	
GAEC1-F	5'-GAG AGG TCA GGG CAG TCC-3'
GAEC1-R	5'-TTT TTT TTT TAG ACA GAG TCT TG-3'

The PCR product was then electrophoresed in a 2% agarose gel and visualized under ultraviolet. The intensities of the PCR products were measured by

densitometric analysis using the Quantity One program (Bio-Rad). The intensities of the target PCR product were normalized against the β-Actin PCR product of each sample.

3.8.3.2. Quantitative PCR analysis (qPCR)

The expression levels of *JK-1*, CCL5 or *IGFBP5* in the corresponding tested cells were analyzed by qPCR analysis. qPCR was performed using Go Taq® qPCR Master Mix (Promega, USA) and Thermo Scientific PikoReal Real-Time PCR System (Thermo Scientific, USA) according to manufacturer's protocol. cDNA (~2 μg) produced by reverse transcription from the RNA was amplified after 2 mins of initiation at 95℃; 40 cycles of denaturation at 95℃ for 15 secs and annealing at 60℃ for 1 min, followed by a final extension at 60℃ for 30 secs, using a specific PCR primer pair for the *JK-1 , CCL5 or IGFBP5* gene (IDT) and the specific *β-Actin* gene primers (IDT) acting as an internal control for normalizing the cDNA quantity. Primers used in qPCR analysis are shown as following:

β-Actin	
β-Actin-Fwd-1	5'-ACC TTC TAC AAT GAG CTG CG-3'
β-Actin-Rev-1	5'-CCT GGA TAG CAA CGT ACA TGG-3'
JK-1	
JK-1- L1	5'-ATG GCA TGG GAA CAA ATG AT-3'
JK-1- R1	5'-TGC TGC ACA CCC TCT AAC TG-3'
CCL5	
CCL5-L1	5'-CGT GCC CAC ATC AAG GAG-3'
CCL5-R1	5'-GGA CAA GAG CAA GCA GAA A-3'
IGFBP5	
IGFBP5- L8	5'-AACGAAAAGAGCTACCGCGA-3'
IGFBP5-R8	5'-CCGACAAACTTGGACTGGGT-3'

Data were obtained and analyzed using PikoReal™ Software 2.0 (Thermo Scientific, USA)

3.8.4. cDNA microarray analysis

Total RNA was extracted from 2×10^8 cells of (1) parental KYSE150, KYSE150-pcDNA3.1-GAEC1, KYSE150-pSilencer-P3-4 and KYSE150-pSilencer-ve; (2) SLMT-1 and SLMT-1/CDDP1R or (3) KYSE150 treated with 91b1 at 9.5μg/mL and DMSO (0.05%, Sigma-Aldrich, USA) for 48hours using RNeasy Mini Kit(Qiagen) as previously described.

The cDNA microarray analysis and the associated quality control using Human Genome U133 Plus 2.0 arrays(Affymetrix) were performed in the Centre for Genomic Sciences of the University of Hong Kong according to the Affymetrix's

protocol.

The RNA integrity was measured by the ratio of 28S/18S ribosomal RNA using Agilent 2100 Bioanalyzer. cDNA was synthesized from 1μg of total RNA using reverse transcription kit (Invitrogen). Biotin labelled-cRNA was produced by *in vitro* transcription kit (Invitrogen) and purified by RNeasy mini columns (Qiagen). About 15 μg denatured cRNA was hybridized to each Human Genome U133 Plus 2.0 array (Affymetrix) and then stained with a streptavidinphycoerythrin conjugate and the signals were detected with GeneArray scanner (Agilent). The microarray signals were analyzed by using Agilent Genespring GX and Affymetrix GeneChip Operating Software. The signals of the differentially expressed genes in the study samples were compared with the corresponding controls. The threshold levels of the corresponding up- or down-regulated genes with ≥ 2 folds were included for the analyses.

3.9. Cell Proliferation Assay

Cell proliferation analysis was performed on (1) parental KYSE150, KYSE150-pSilencer-P3-4 and KYSE150-pSilencer-ve; (2) parental KYSE70, KYSE70-shcon and KYSE70-sh1 or (3) SLMT-1 and SLMT-1/CDDP1R cultured in medium containing or without 9.1μg/mL cisplatin. The cells were harvested by

trypsinization and the cell number was counted by hemocytometer under microscope. The cells were plated in flat-bottom 96-well plate in 100μL of respective culture medium at a density of 1,000 cells per well. For study of CDDP-resistance, SLMT-1 and SLMT-1/CDDP1R were plated in flat-bottom 96-well plate in 100μL of culture medium without CDDP for cell seeding. For wells examining the proliferation in the condition with CDDP, the medium was replaced with medium containing 9.1μg/mL CDDP 24 hours after seeding of cells.

CellTiter-96 AQueous One Solution Cell Proliferation (MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]) Assay kit (Promega, USA) was used for the quantization of cell viability. MTS working solution was prepared by diluting five times with autoclaved PBS before use. 100μL of the MTS working solution was added to each well after removal of culture medium every 24-hour and incubated at 37 ℃ for 2.5hours, The cell viability was then determined by measuring the absorbance of the well at 490nm using microplate reader (Ultramark, BioRad). Relative growth (related to cell viability at 0 hours) of each cell line was calculated by $\frac{[A]_T}{[A]_{T0}}$. Where $[A]_T$ is the absorbance at different time points and $[A]_{T0}$ is the absorbance at 0 hours. This assay was performed in triplicate.

3.10. Cell cycle analysis

Parental KYSE150, KYSE150-pSilencer-P3-4 and KYSE150-pSilencer-ve cells were harvested from culture flasks at 80% confluence as previously described. The cells were washed with cold PBS, followed by fixing with 1 mL of 70% ethanol for 24 hours at -4°C. The cells were washed with PBS after fixing and then stained with formulated propidium iodide (Sigma-Aldrich) staining solution (20μg/mL propidium iodide, 0.1%v/v Triton X-100, 200μg/mL RNase A) at 37°C for 30 mins. The samples were analyzed by BD FACSCalibur flow cytometer. Different fractions of cell cycles were analyzed using the Modfit LT software (Verity Software House).

3.11. MTS cytotoxicity assay

Cytotoxic effect of (1)CDDP or (2) 91b1 and CDDP on (1)SLMT-1, SLMT-1/CDDP1R, SLMT-1-IGFBP5, SLMT-1-pcMV3, SLMT-1-R-IGFBP5 , SLMT-1-R-pcMV3 and SLMT-1/IGFBP5-siRNA (2) KYSE150, KYSE510, KYSE450, KYSE30, HKESC-4, NE-3 and HEK293 were examined by CellTiter-96 AQueous One Solution Cell Proliferation Assay (Promega, USA).

The cells were harvested by trypsinization as described in section 3.1.1. and the cell number was counted by hemocytometer under microscope. The cells were

plated in flat-bottom 96-well plate in 100ul of respective culture medium at a density of 5,000 cells per well. 24 hours after seeding the cells, the cells were incubated with 40, 20, 10, 5, 2.5, 1.25 and 0μg/mL of CDDP or 91b1 and 0.01% of DMSO and incubated for 48 hours. After 48 hours of incubation, medium were removed without disrupting the seeded cells. 100μL of working MTS solution was added to each well and reading were taken with microplate reader (Ultramark, BioRad) as described in Section 3.9. This assay was performed in triplicate.

3.12. Trans-well matrigel invasion assay

Invasion of tumor cells was evaluated using chambers with matrigel-coated membrane (8-μm pore size ,BD Biocoat, Corning) in 24-well plate. The lower chamber was filled with RPMI1640 medium containing 10% FBS with recombinant human CCL5 (rhCCL5, Abnova, Taiwan) at concentration of 0ng/mL, 50ng/mL, 100ng/mL and 500ng/mL. KYSE30 was cultured in 200ul serum free RPMI1640 medium in the upper chamber at a density of $2.5x10^5$ cells/mL. Same amount of cells were cultured to uncoated membrane (8-μm pore size) chamber as control. After 24 hours, the uninvaded cells on the upper chamber were scraped off with a cotton swab. The transmembrane cells which migrated to the opposite side of the membrane were fixed in 100% methanol for 10 mins and stained with

0.5% crystal violet solution (0.5g crystal violet in 75mL methanol and 25mL ddH_2O) after washing twice with PBS. The transmembrane cells were counted under microscope in 5 random fields at magnification of 100×. The percent invasion was calculated as shown below % invasion = $\frac{\text{mean number of cell invading through matrigel coated membrane}}{\text{mean number of cells migrating through uncoated membrane}}$ X 100

3.13. Immunohistochemical (IHC) staining

Tissue microarrays (TMA) of paraffin-embedded ESCC specimens and esophageal epithelia were constructed with a Beecher Instruments tissue microarraryer (Beecher Instruments, Silver Spring, USA) as previously described (Yuen, H. *et al.*, 2007) and were kindly provided by Dr. K. W. Chan. The archival paraffin-embedded ESCC tissues were used under the ethical guidelines in the Department of Pathology of The University of Hong Kong. Dewaxed paraffin sections (8μm) were immunostained using the streptavidin-biotin-peroxidase complex method. As pretreatment, microwave-based antigen retrieval will be performed in 10 mM citrate buffer (pH 6.0). Calpain 10 rabbit polyclonal antibody(0.03 mg/mL; 1:50; Sigma-Aldrich); JK-1 rabbit polyclonal antibody(0.1mg/mL;1:5, Santa Cruz Biotech, Inc) or CCL5 mouse monoclonal antibody (1:100; 1mg/mL, Abnova) were applied to the tissues at the respective

dilution factors for overnight incubation at 4°C. The photos of staining sections were examined and graded according to the percentage of positively stained tumor cells. The grades (0, I-III) were defined as follows: Grade 0: less than 5%, Grade I: 5% to less than 25%, Grade II: 25% to less than 50% and Grade III: more than 50%. For each tissue sample, the tissue core with the highest grade was selected for subsequent statistical analysis. The high expression group combined those tumors with Grade II or III and the low expression group combined those tumors with Grade 0 or I.

3.14. Immunostaining

(1) For study of cisplatin-resistance, nuclear protein expression level of Myc in SLMT-1, SLMT-1/CDDP1R, SLMT-1-pcMV3, SLMT-1-R-pcMV3, SLMT-1-IGFBP5 and SLMT-1-R-IGFBP5 cell lines were examined using immunostaining to evaluate the transfection efficiency of Myc tagged IGFBP5/pcMV3-C-Myc and Myc tagged pcMV/hydro-negative control.

(2) For study of 91b1, immunostaining was used to evaluate CCL5 protein expression level in KYSE150, KYE510, KYSE450, KYSE30, KYSE520, HKESC-3, HKESC-4, SLMT-1 and KYSE150 treated with vehicle DMSO, 91b1 with concentration of 6.5μg/mL, 9.5μg/mL and 21μg/mL for 48hours.

About $5x10^6$ of all tested cells were harvested by cell scrapper (SPL, Korea) and washed with PBS for two times. The washed cell pellets were resuspended and incubated in 10% formalin buffered in phosphate solution at room temperature overnight. After overnight incubation, cell pellets were formed by centrifugation at 1000g. Formalin-fixated cells were embedded into paraffin blocks. Dewaxed paraffin sections (8μm) were pretreated as previously described (Section 3.13). CCL5 mouse monoclonal antibody (1:100; 1mg/mL, Abnova) or Myc mouse antibody (1:8000; 1mg/mL, Sino Biological Inc.) were applied to the tissues at the respective dilution factors for overnight incubation at 4℃. The photos of staining sections were examined according to the percentage of positively stained cells.

3.15. *In vitro* wound healing assay

KYSE70 cells, KYSE70-shcon cells and KYSE70-sh1 cells were cultured in 6-well plates at $1X10^6$ cells/well. The cells were allowed to adhere and grow to confluent monolayers in the presence of its culture medium. The monolayers were wounded in a straight line across the well with a 10-μL standard pipette tip. The wounded monolayers were washed with PBS to remove cell debris and incubated for another 120 hours. The wounds were examined and photographed using an inverted phase contrast microscope with digital camera (1X71, Olympus) at zero

time point and 120-hour.

3.16. Colony formation assay in soft agar

The soft agar plate is composed of base layer with 0.8% agar and top layer with 0.4% agar containing 10^5 cells. The base layer was prepared with serum free RPMI medium and 1.6% autoclaved agar in a 1:1 ratio. After solidification of the base layer at 4°C, the plate was pre-warmed at 37°C before setting the top layer. KYSE70 cells, KYSE70-shcon cells and KYSE70-sh1 cells were harvested from culture flasks by trypsinization. Cell suspensions containing 10^5 each kind of cells were mixed with 0.8% agar in a 1:1 ratio respectively. These cell-containing agar was placed onto base layer in 6-well plate at 1.5mL per well. 1.5 mL of culture medium was placed onto the top layer after solidification of top layer. The plate was incubated at 37℃ and 5% CO_2 for 4 weeks. Culture medium was replaced every three days. The number of colonies in each well was counted under light microscope (CKX41, Olympus) after 4 weeks of incubation. Photos were taken using light microscope (CKX41, Olympus) with digital camera (DP71, Olympus). The assay was performed in triplicate.

3.17. Foci formation assay

KYSE70 cells, KYSE70-shcon cells and KYSE70-sh1 cells were seeded separately to 6-well plates at a density of $1x10^5$ per well. The cells were maintained in their culture medium and incubation condition as described in section 3.1 for 2 weeks. Culture medium was replaced every two days. The cells were washed with 1 mL of PBS and stained with 1 mL of crystal violet (0.05%v/v, Sigma-Aldrich, China) to observe the foci.

3.18. Morphological study

Morphologies of SLMT-1 cells and SLMT-1/CDDP1R cells in culturing medium with or without 9.1μg/mL cisplatin were observed under microscope at 0, 48 and 72 hours. Photos of cells were captured using light microscope (CKX41, Olympus) with digital camera (DP71, Olympus) at 200×magnification.

3.19. Enzyme-linked immunosorbent assay (ELISA)

Protein levels of CCL5 in 91b1-treated, DMSO-treated and untreated ESCC cells were measured using RANTES (CCL5) Human SimpleStep ELISA™ Kit (Abcam). Cells received no treatment, DMSO-treatment or 91b1-treatment (in concentration of 6.5μg/mL, 9.5μg/mL and 21μg/mL) were collected by cell

scrapper after 48 hours. ELISA was conducted according to the manufacture's instruction. Absorbance was measured using microplate reader (Ultramark, BioRad) at a wavelength of 450 nm. Total protein concentrations of the samples were determined using Pierce™ BCA Protein Assay kit (ThermoFisher, USA) according to the manufacture's manual for normalization.

3.20. Statistical analysis

Two-tailed t-test or one-way analysis of variance (ANOVA) was used to determine the statistical significance of differences between groups. Comparative ΔΔCt method was applied for relative quantification in qPCR analysis (Schmittgen, T. D. and Livak, K. J., 2008). The statistical significance of the differences in calpain 10, JK-1 and CCL5 expression between tumor, premalignant and non-tumor tissues in IHC analysis were evaluated by Student's *t* test. The correlations of the IHC signals with clinicopathological parameters were performed using Pearson's correlation. The statistical significance of the correlations was determined by Student's *t* test or chi-square test or fisher's exact test. Patients' survival curve was plotted by Kaplan-Meier methods and analyzed by Cox multi-variant analysis and Wilcoxon's signed-rank test.

Statistical analysis was performed using SPSS Ver. 20 (SPSS, Chicago, IL, United

States) or GraphPad Prism 5. Differences were considered statistically significant when the relevant p values were < 0.05.

Chapter 4 Results

4.1. Study of downstream regulation of oncogene *GAEC1* and its clinical significance in ESCC

4.1.1. *GAEC1* expression analysis by PCR

By using multiplex semi-quantitative RT-PCR and densitometry analysis, it was confirmed that *GAEC1* is overexpressed in ESCC cell line KYSE150 over non-tumor esophageal epithelial cell line NE-1.

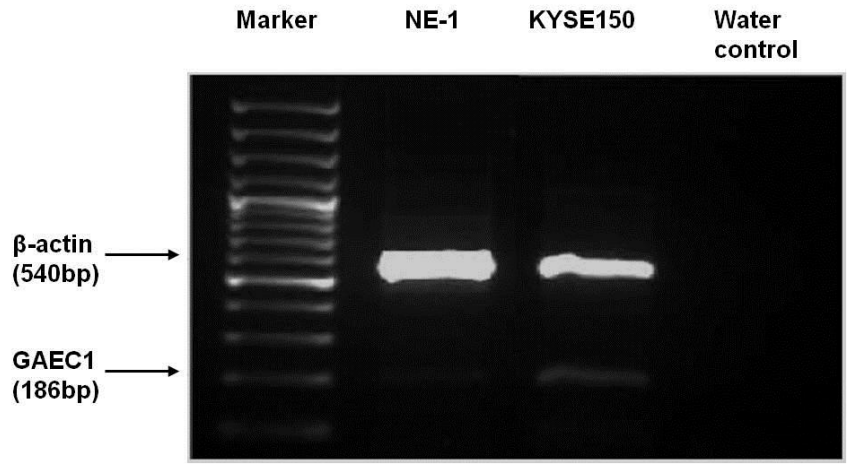


Figure 4.1 Expression of *GAEC1* in ESCC cell line KYSE150 and non-tumor esophageal epithelial cell line NE-1.
Water was used as a negative control; β-actin was used as a loading control.

As *GAEC1* was found overexpressed in KYSE150, KYSE150 was used as the cell line model for the study of suppression of *GAEC1* expression by siRNA expression vector pSilence-P3-4.

The expression level of *GAEC1* in pSilencer-P3-4 transfected KYSE150 cells (KYSE150-pSilencer-P3-4) was determined by comparing with the parental and pSilencer-negative control vector transfected cells (KYSE150-pSilencer-ve) using densitometry measurement. The results indicated that the KYSE150- pSilencer-P3-4 cells showed a down-regulation of *GAEC1* expression compared with the parental cells and KYSE150-pSilencer-ve. The comparison of the band intensities among the samples by densitometry measurement showed that the *GAEC1* expression level was down-regulated in KYSE150- pSilencer-P3-4 cells by about three folds.

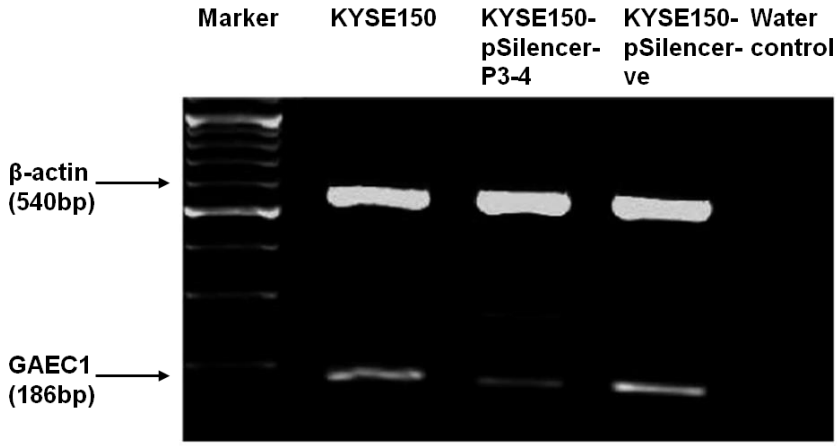


Figure 4.2 Expression of *GAEC1* in KYSE150, KYSE150-pSilencer-P3-4 and KYSE150-pSilencer-ve.

4.1.2. Cell proliferation assay

To study the effects on cell proliferation with suppressed *GAEC1* expression, cell proliferation assay using MTS was performed on KYSE150-pSilencer-P3-4, KYSE150-pSilence-ve and parental KYSE150 cells. The results demonstrated that KYSE150 cells with down-regulated *GAEC1* showed an obvious reduction in proliferation rate compared with the parental and control-vector transfected cells.

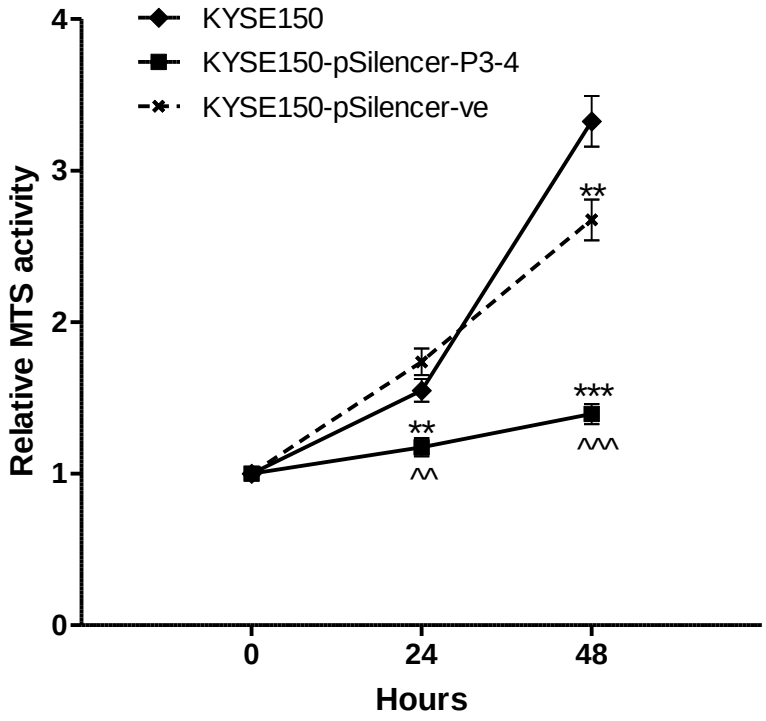


Figure 4.3 MTS cell proliferation assay for KYSE150, KYSE150-pSilencer-P3-4 and KYSE150-pSilencer-ve.
Compared to KYSE150, ** $p<0.01$ and *** $p<0.001$;
Compared to KYSE150-pSilencer-ve, ^ ^$p<0.01$. and ^^^ $p<0.001$.

4.1.3. Cell cycle analysis

To extend the understanding of slower proliferation rate in KYSE150-pSilencer-P3-4, cell cycle analysis was conducted to reveal the cell cycle distribution of KYSE150-pSilencer-P3-4, KYSE150 parental and KYSE150-pSilencer-ve (Figure 4.4). Approximately 50% increase in apoptosis population was observed in *GAEC1*-suppressed cells, KYSE150-pSilencer-P3-4 cells, compared with parental KYSE150 cells and KYSE150-pSilencer-ve cells. This indicated that suppression of *GAEC1* expression lead to slower proliferation rate by causing increased apoptosis.

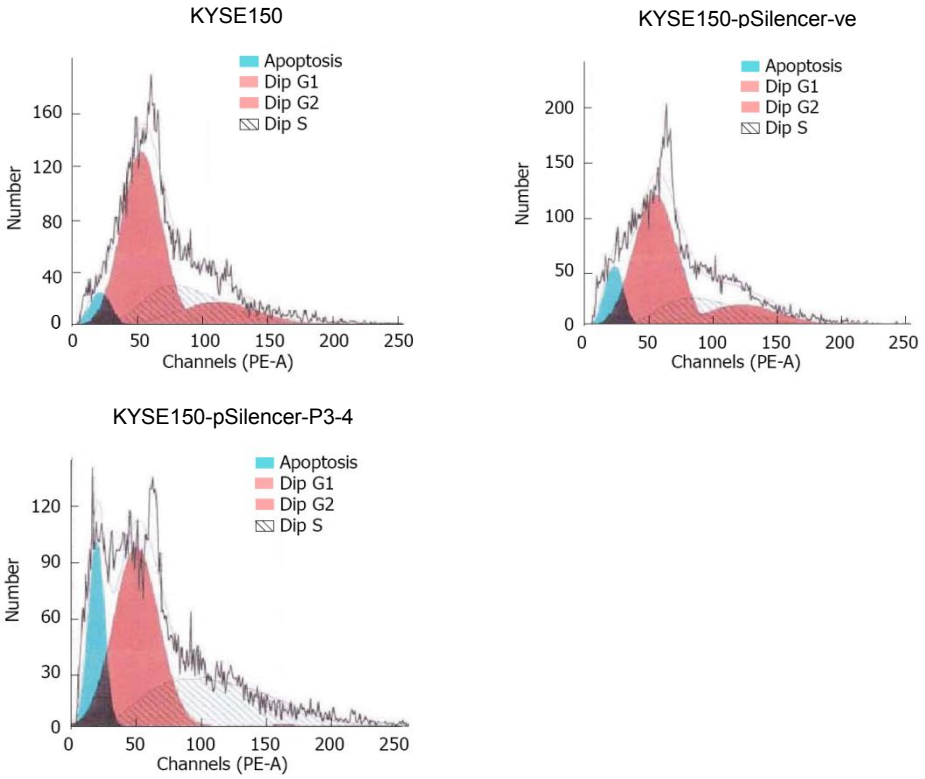


Figure 4.4 Flow cytometry analysis for KYSE150, KYSE150-pSilencer-ve and KYSE150-pSilencer-P3-4

4.1.4. cDNA microarray analysis

To identify the downstream candidate genes which are regulated by the suppressed *GAEC1* expression, cDNA microarray analysis was performed using the Human Genome U133 Plus 2.0 array (Affymetrix) which comprises of over 47,000 transcripts and variants in each chip.

Genes with expression signal of more than 2-fold increase or decrease in KYSE150-pcDNA3.1-*GAEC1* cells compared with those in parental KYSE150 and no significant fold change in pSilencer-negative control vector transfected cells were the candidate genes. Candidate genes were listed according to fold change of expression signal increase or decrease in KYSE150-pSilencer-P3-4 cells compared with KYSE150 parental.

Ten genes with more than 5-fold decrease were identified (Table 4.1). On the other hand, nine genes with more than 3-fold increase were identified (Table 4.2). Trinucleotide repeat containing 6C (*TNRC6C*) had the highest level, over 7 folds, of upregulation. Calpain 10 (*CAPN10)* showing the highest fold change (downregulaed by 15.3 times; Table 4.1) in microarray analysis was followed up by IHC analysis (section 4.1.5)

Table 4.1 List of more than 5-fold downregulated genes induced by stable *GAEC1* knockdown in KYSE150 cells compared with KYSE150 cells.

Probe set ID	Gene title	Down-regulation with transfected pSilencer P3-4	Up-regulation with transfected pcDNA3.1-*GAEC1*	pSilencer -ve control
221040_at	Calpain 10	15.3033010	2.1643467	1.0476209
1561417_x_at	Not assigned	12.1628650	2.0130675	1.1347373
1562828_at	Not assigned	9.9816000	2.6808436	1.1661105
229929_at	splA/ryanodine receptor domain and SOCS box containing 4	8.3420770	2.2365010	1.1751518
235209_at	Chromosome 8 open reading frame 84	7.6294910	2.1356385	1.1450081
220090_at	Cornulin	7.6125007	2.3401918	1.1664450
242713_at	Not assigned	7.3507795	2.0578532	1.1959343
224499_s_at	Activation-induced cytidine deaminase	5.8917794	3.3146940	1.1664389
229543_at	Not assigned	5.3493247	2.0206234	1.0065930
242064_at	Sidekick homolog 2 (chicken)	5.0322995	2.8469403	1.0170712

Table 4.2 List of more than 3-fold upregulated genes induced by stable *GAEC1* knockdown in KYSE150 cells compared with l KYSE150 cells.

Probe set ID	Gene title	Up-regulation with transfected pSilencer P3-4	Down-regulation with transfected pcDNA3.1-*GAEC1*	pSilencer -ve control
1561041_at	Trinucleotide repeat containing 6C	7.5979643	2.1234870	1.0152589
216787_at	Not assigned	5.3369575	2.2657390	1.1658608
206725_x_at	Bone morphogenetic protein 1	4.8828310	2.7046654	1.1015952
206276_at	Lymphocyte antigen 6 complex, locus D	4.7652740	2.7379642	1.0686288
1560482_at	Not assigned	4.2348604	3.0122058	1.1875614
211362_s_at	Serpin peptidase inhibitor, clade B (ovalbumin), member 13	4.0584164	2.4208739	1.0186443
216491_x_at	Immunoglobulin heavy constant mu	3.4819565	2.8060850	1.0186309
238415_at	Not assigned	3.2034543	2.9523630	1.0138865
241028_at	RPGRIP1-like	3.0331728	2.3752263	1.0001514

4.1.5. Immunohistochemical staining of CAPN10

14 out of 132 tumors were found to belong to the high expression group of CAPN10 expression which counted for 10.61% of total.

Representative examples of immunohistochemical staining of CAPN10 are shown in Figure 4.5.

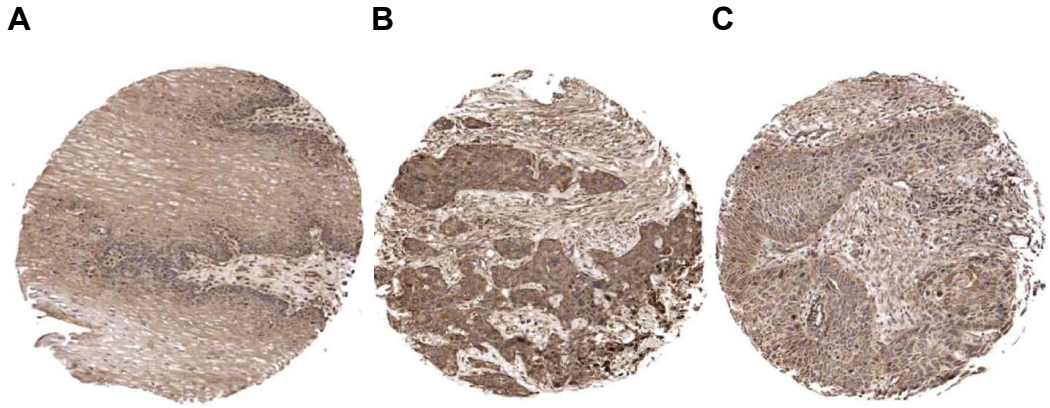


Figure 4.5 Representative photos of immunohistochemical staining of CAPN10 (original magnification×100)
A: Non-tumor esophageal epithelial tissue showing weak CAPN10 staining;
B: ESCC tissue showing strong CAPN10 staining;
C: ESCC tissue showing weak CAPN10 staining in tumor.
CAPN10 was mainly localized in the cytoplasm of the cancer cells

4.1.6. Clinicopathological correlation of CAPN10 to ESCC patient specimens

Correlation between expression level of CAPN10 and clinicopathological features are summaries in Table 4.3. There was no significant correlation of any clinicopathological features with the expression level of CAPN10.

However, there was significant difference in the median survival of patients with different *CAPN10* expression level (Figure 4.6). For patients with high expression level the median survive was 38 months whereas that for patients with low expression level was 13 months, The survival range was from 0.72 to 65.15 months. The difference was significant on both univariant and multi-variant analysis with P-values of 0.032 and 0.035 respectively.

Table 4.3 Relationship between CAPN10 expression and clinicopathological features

Characteristics	Patients	Low expression	High expression	P value
Age, yr (mean ± SD)	132	65.64 ± 10.55	63.50 ± 13.39	0.572
Gender				0.086
Male	102	94	8	
Female	30	24	6	
TNM stage				0.762
0/I/II	18	15	3	
III/IV	83	72	11	
Tumor depth				0.729
T1-3	79	67	12	
T4	22	20	2	
Lymph node metastasis				0.363
N0	32	26	6	
N1	69	61	8	
Distant metastasis				1.000
M0	66	57	9	
M1	35	30	5	
Differentiation				0.459
Well	16	15	1	
Moderate	59	51	8	
Poor	26	21	5	

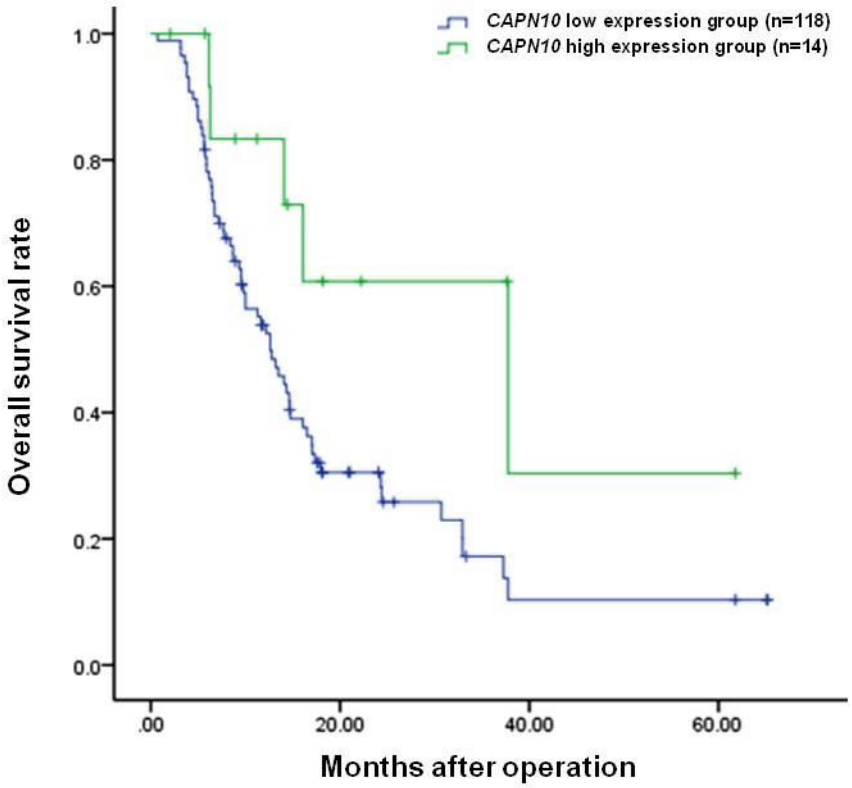


Figure 4.6 Overall 5-year survival rates as determined by the expression level of CAPN10 in ESCC patients.

Low expression group of CAPN10 in ESCC patients showed a significantly lower 5-year survival rate than those of high expression group.

4.2. Study of reversal of tumor transforming properties of *JK-1*

4.2.1. *JK-1* expression in cell lines by qPCR

Expression of *JK-1* in four ESCC cell lines KYSE70, KYSE150, KYSE510 and SLMT-1 and a non-tumor esophageal cell line NE-3 were examined by qPCR analysis. The relative *JK-1* expression was determined by comparing with *JK-1* expression in NE-3 (Figure 4.7). KYSE150 and SLMT-1 showed similar *JK-1* expression level as NE-3 while KYSE70 and KYSE510 showed significantly higher *JK-1* expression level than NE-3. The expression levels of KYSE70 and KYSE510 were approximately 15 times and 5 times of level in NE-3. KYSE70, showing the highest *JK-1* expression level, was therefore selected for demonstrating the reversal of tumor transforming properties of *JK-1* in the current study.

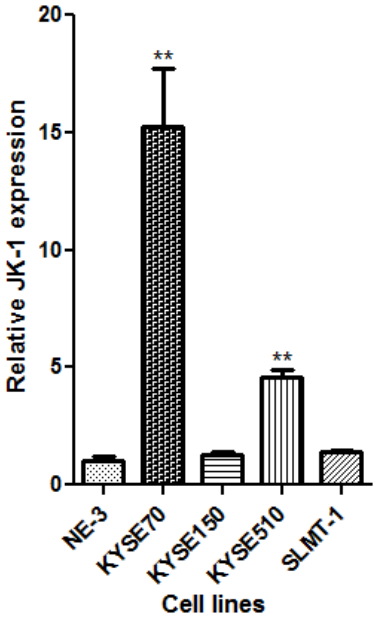


Figure 4.7 Relative expression levels of *JK-1* in KYSE70, KYSE150, KYSE510 and SLMT-1 and NE-3.

Relative JK-1 expression levels were determined by comparing with NE-3 cells, after normalized with expression of β-actin. ** $p<0.01$

4.2.2. Suppression of *JK-1* expression by *JK-1* siRNA expression vector

To suppress the expression of *JK-1*, siRNA expression vector specific for *JK-1 (JK-1*- siRNA-psiNU6) was transfected into KYSE70. After puromycin selection, a stably *JK-1*-siRNA expressing cell line, KYSE70-sh1, was established. And mock vector was also transfected into KYSE70 to generate KYSE70-shcon as a control cell line. *JK-1* expression in KYSE70 parental, KYSE70-shcon and KYSE70-sh1 were examined by qPCR analysis (Figure 4.8). There was no significant difference in *JK-1* expression level in KYSE70-shcon and KYSE70 parental. And KYSE70-sh1 showed significant reduction in relative expression level of *JK-1* in KYSE70-sh1 compared with KYSE70-shcon. The suppression on *JK-1* expression by the siRNA expression vector was resulted and KYSE70-sh1 cells were thus suitable for evaluation of reversal of *JK-1* transforming properties in the present study.

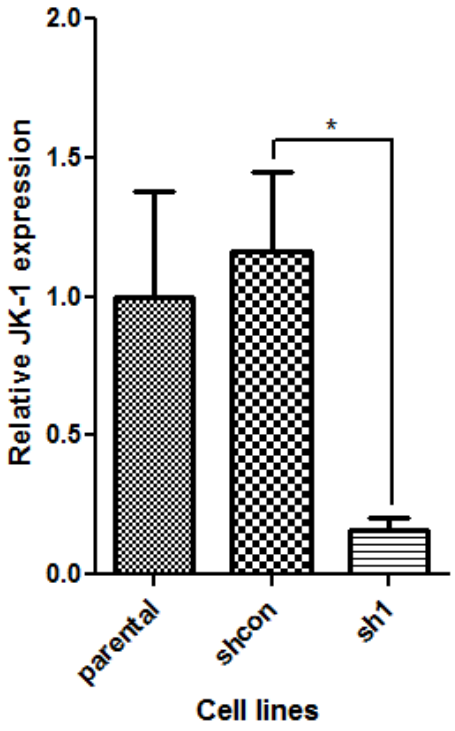


Figure 4.8 Relative expression levels of *JK-1* in KYSE70, KYSE70-shcon and KYSE70-sh1.

Relative JK-1 expression levels were determined by comparing with KYSE70 parental cells, after normalized with expression of β-actin. * $p<0.05$

4.2.3. Cell proliferation assay

To study the effects on cell proliferation with suppression of *JK-1*, cell proliferation assay was performed on KYSE70, KYSE70-shcon and KYSE70-sh1 using MTS. The proliferation curve (Figure 4.9) showed that KYSE70 cells with downregulated *JK-1* expression (KYSE70-sh1) showed a significant reduction in proliferation rate starting from 72-hour to 120-hour compared with control-vector transfected cells(KYSE70-shcon), and from 96-hour to 120-hour compared with the parental cells. And the transfection of control vector into KYSE70 cells did not cause remarkable change on the cell proliferation rate.

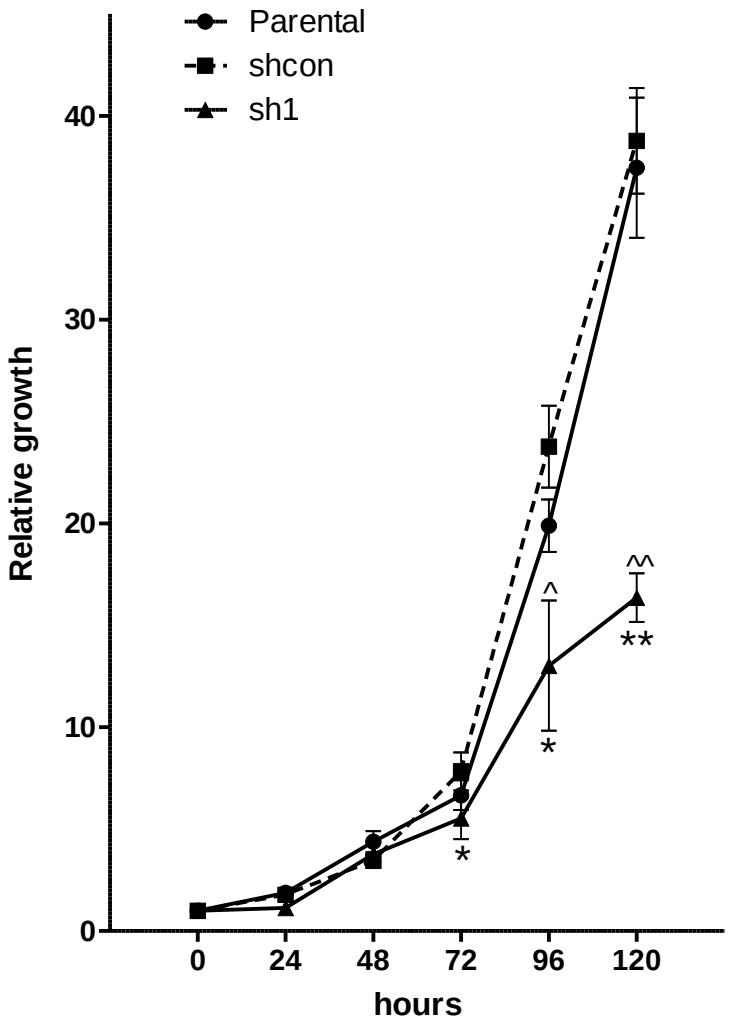


Figure 4.9 MTS cell proliferation assay of KYSE70, KYSE70-shcon and KYSE70-sh1.

Compared to KYSE70 ^ $p<0.05$ and ^^ $p<0.01$; Compared to KYSE70-shcon, * $p<0.05$. and ** $p<0.01$.

4.2.4. *In vitro* wound healing assay

In vitro wound healing assay was performed on KYSE70 parental, KYSE70-shcon and KYSE70-sh1 cells (Figure 4.10). The cells were grown to 95% confluence and the monolayer was scratched with pipette tip. After 120 hours of incubation, KYSE70 and KYSE70-shcon cells generally got more cells migrated into the scratched area than KYSE70-sh1.

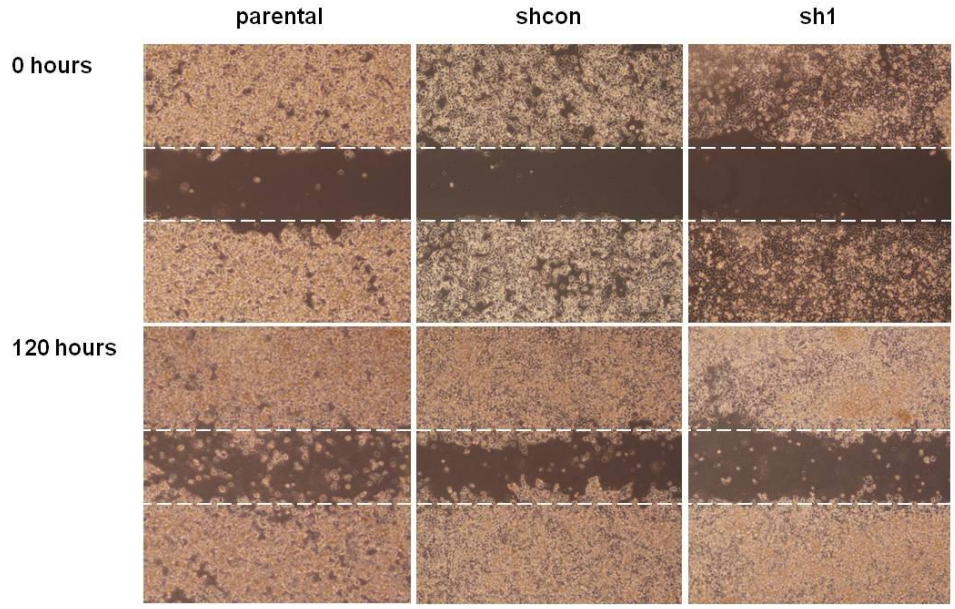


Figure 4.10 *In vitro* wound healing assay for KYSE70 parental, KYSE70-shcon and KYSE70-sh1 cells. (original magnification of x100)

4.2.5. Colony formation assay in soft agar

The anchorage-independent growth ability of KYS70, KYSE70-shcon and KYSE70-sh1 were examined in soft agar. Photos of colonies formation status were captured and shown in Figure 4.11 and the numbers of colonies formation were shown in Figure 4.12.

KYSE70 parental cells and KYSE70-shcon cells showed their ability to form colonies in soft agar. While no colonies were observed for KYSE70-sh1 cells in soft agar. There was significant difference in number of colonies formed for KYSE70-sh1 compared with KYSE70 and KYSE70-shcon. This indicated the loss of anchorage-independent growth ability for KYSE70-sh1 cells.

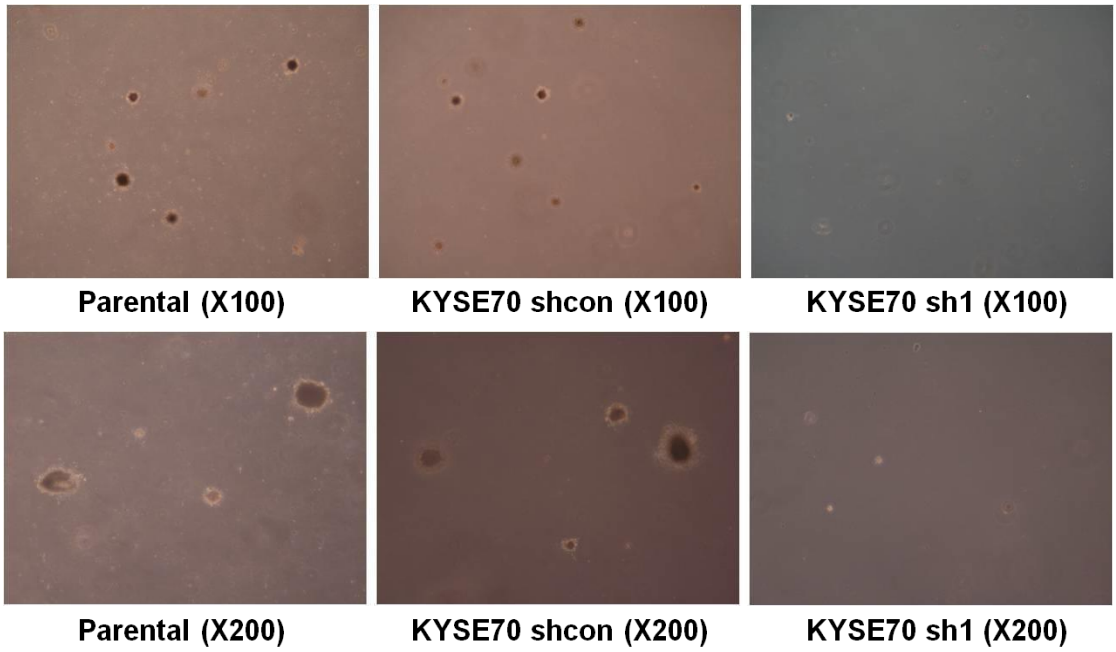


Figure 4.11 Representative photos for colony formation ability of KYSE70 parental, KYSE70-shcon and KYSE70-sh1 in soft agar.
(original magnification ×200)

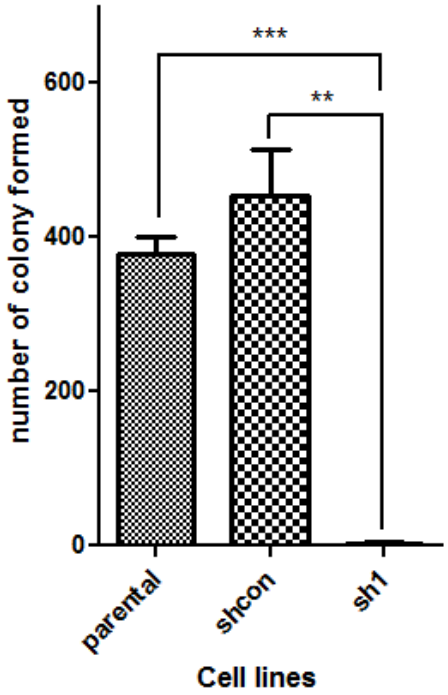
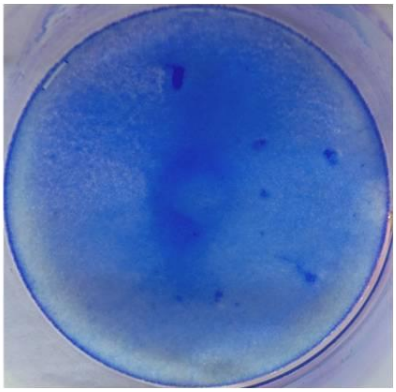


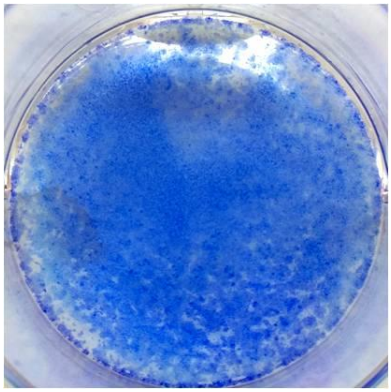
Figure 4.12 Number of colonies formed by KYSE70 parental, KYSE70-shcon and KYSE70-sh1 in soft agar after 4 weeks. Results were expressed with mean±SD from triplicate experiments. ** p<0.01 and ***p<0.001

4.2.6. Foci formation assay

Cells of KYSE70, KYSE70-shcon and KYSE70-sh1 were allowed to grow for 10 days. Cells of KYSE70 and KYSE70-shcon showed anchorage-dependent growth ability that foci were widely formed while no foci were observed for KYSE70-sh1. Loss of anchorage-dependent growth ability was reversed in KYSE70-shl.



KYSE70 parental



KYSE70-shcon

KYSE70-sh1

Figure 4.13 Foci formation of KYSE70 parental, KYSE70-shcon and KYSE70-sh1 cells. KYSE70-sh1 cells showed the obvious reduction in foci formation. Photos were taken 10 days after cells seeding.

4.2.7. *JK-1* protein expression in ESCC patient samples

4.2.7.1. Correlation with histopathological features

JK-1 expression level was examined by immunohistochemical staining in 15 non-tumor tissues, 16 pre-malignant tissues (hyperplasia and dysplasia) and 26 tumor tissues. Protein expression of JK-1 in nuclei was examined as suggested in previous study on colorectal cancer (Kasem, K. *et al.*, 2014). IHC staining photos of JK-1 in non-tumor, pre-malignant and tumor tissues were shown in Figure 4.14.

Table 4.4 summarized the protein expression level of JK-1 in non-tumor, pre-malignant and tumor tissues. 93.3% (14/15) of non-tumor tissues, 87.5% (14/16) of pre-malignant tissues and 26.9% (7/26) of tumor tissues showed high expression for JK-1. A trend was observed that the frequencies of high levels of JK-1 protein expression were higher at the early stage and reduced in mature tumors ($p<0.001$).

Table 4.4 Summary of results showing the expression levels of JK-1 in non-tumor, pre-malignant and tumor tissues.

Histopathological features	No. of specimens	High expression	Low expression
Non-tumor	15	14(93.3%)	1(6.7%)
Pre-malignant	16	14(87.5%)	2(12.5%)
Tumor	26	7 (26.9%)	19(73.1%)

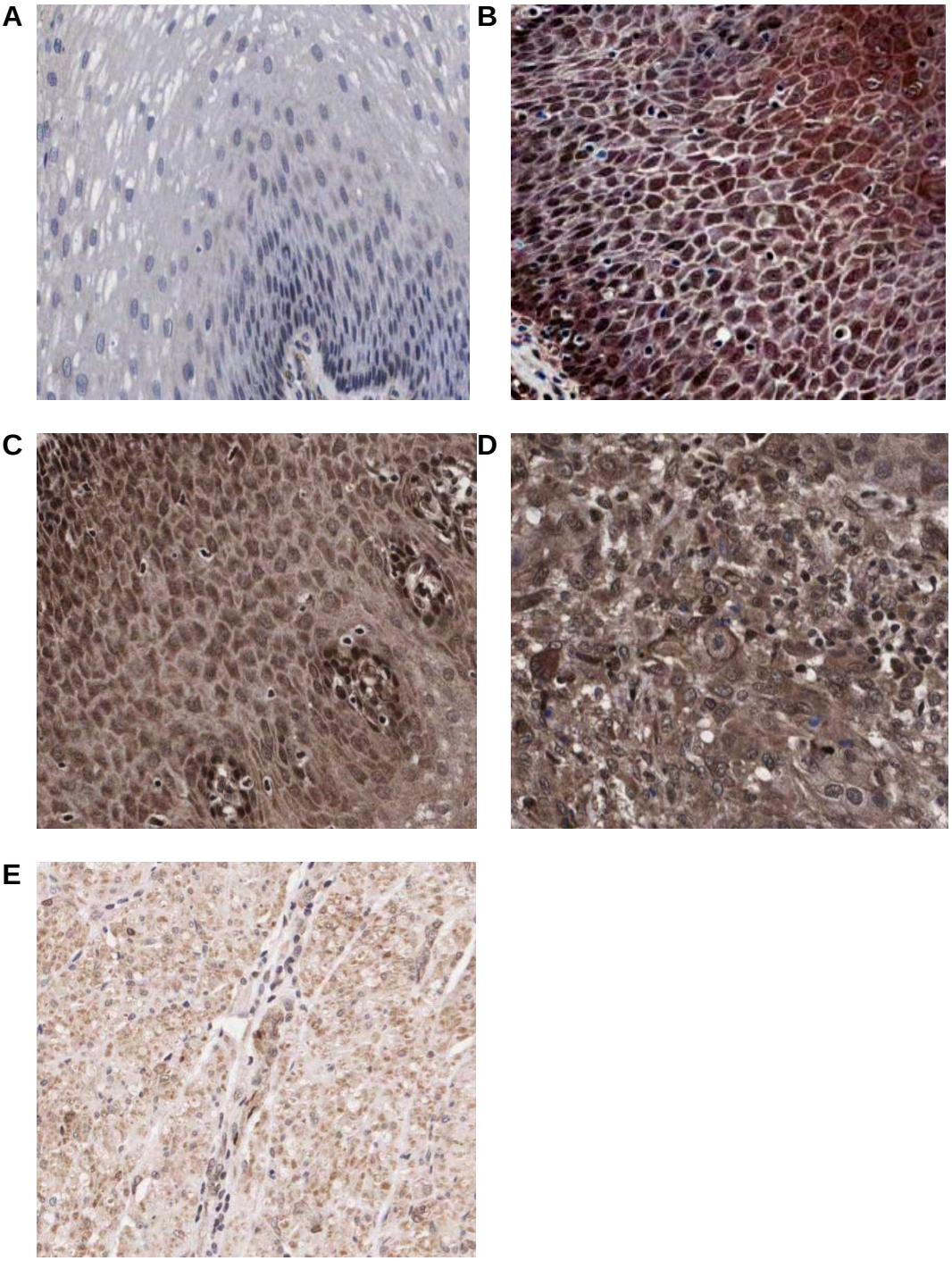


Figure 4.14 IHC staining photos of (A) non-tumor squamous epithelium with low level of JK-1 expression; (B) esophageal dysplasia with high level of JK-1 expression (cytoplasm & nucleus); (C) esophageal hyperplasia with high level of JK-1 expression (cytoplasm & nucleus); (D) ESCC with high level of JK-1 expression (cytoplasm & nucleus); and (E) ESCC with low level of JK-1 expression (cytoplasm & nucleus). (original magnification x400)

4.2.7.2. Correlation with clinicopathological features

Twenty-six ESCC tumor tissue specimens were examined for the correlation of JK-1 protein expression with clinicopathological features using immunohistochemistry. The clinicopathological features are listed in Table 3.2.

There were 30.8% of (8/26) cases in early stage (stage I or II), 69.2% (18/26) in advanced stage (stage III or IV). 37.5% (3/8) of cases in early stage showed high expression for JK-1 while 22.2% (4/18) showed high expression for JK-1 in the advanced stage cases.

According to the degree of differentiation, 34.6% (9/26) of specimens were well-differentiated, 61.5% (16/26) were moderately-differentiated and 3.8% (1/26) was poorly-differentiated. 22.2% (2/9) of well-differentiated specimens, 25% (4/16) of moderately-differentiated specimens and 100% (1/1) of poorly-differentiated specimens belonged to JK-1 high expression group.

Correlations between expression level of JK-1 and clinicopathological features are summarized in Table 4.5. No significant correlation between the clinicopathological features and the expression level of JK-1 was observed from these 26 ESCC cases.

Table 4.5 Relationship between JK-1 expression and clinicopathological features

Characteristics	No. of Patients	High expression	Low expression	P-value
Age, yr (mean ± SD)	26	62.43±13.45	64.05±9.28	0.062
Gender				0.661
Male	17	4 (23.5%)	13 (76.5%)	
Female	9	3 (33.3%)	6 (66.7%)	
TNM stage				0.635
I/II	8	3 (37.5%)	5 (62.5%)	
III/IV	18	4 (22.2%)	14 (77.8%)	
Tumor depth				1.000
T1-3	19	5 (26.3%)	14 (73.7%)	
T4	7	2 (28.6%)	5 (71.4%)	
Lymph node metastasis				0.407
N0	11	4 (36.4%)	7 (63.6%)	
N1	15	3 (20.0%))	12 (80.0%)	
Distant metastasis				1.000
M0	24	7 (29.2%)	17 (70.8%)	
M1	2	0 (0.0%)	2 (100%)	
Differentiation				0.241
Well	9	2 (22.2%)	7 (77.8%)	
Moderate	16	4 (25.0%)	12 (75.0%)	
Poor	1	1 (100%)	0 (0.0%)	

4.2.7.3. Survival analysis

The survival in ESCC patients was correlated with JK-1 expression status using the Kaplan-Meier analysis (Figure 4.15). The median survival months for patients with high nuclear JK-1 expression (n=7) was 8.79 months while that for patients with low nuclear JK-1 expression (n=19) was 9.57 months .

No statistically significant difference was observed for expression in both nuclei and cytoplams (p=0.909)

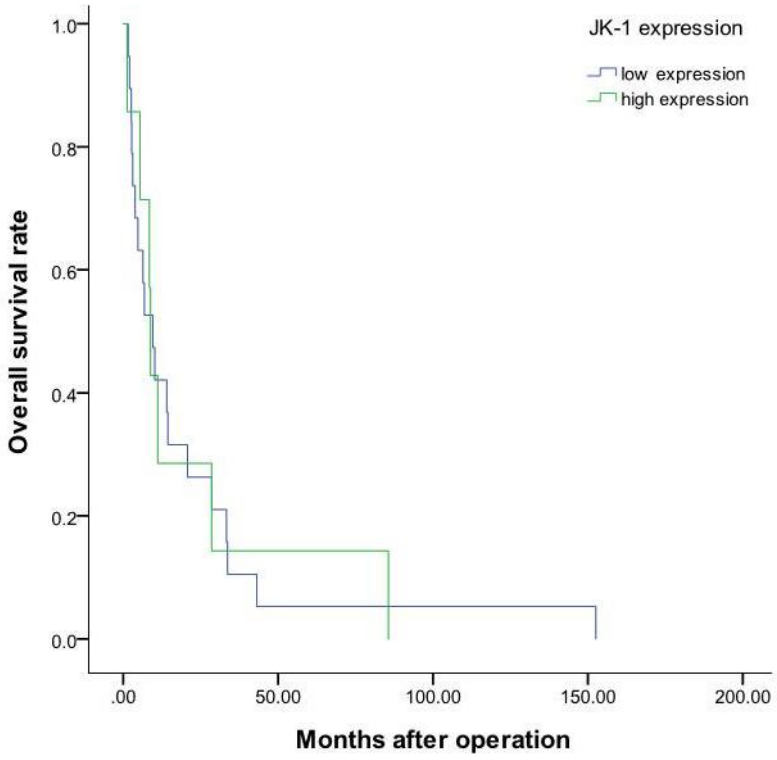


Figure 4.15 Overall survival rates as determined by JK-1 expression level

4.3. Study of reversal of cisplatin-resistance in ESCC cells

4.3.1. Verification of acquiring cisplatin-resistance

4.3.1.1. MTS cytotoxicity assay

MTS assay was performed to characterize the sensitivity of SLMT-1 cells and SLMT-1/CDDP1R cells to cisplatin. As shown in Figure 4.16, SLMT-1/CDDP1R showed remarkably higher relative MTS activity than SLMT-1 after 48-hour treatment with cisplatin at concentration of 2.5 -20μg/mL.

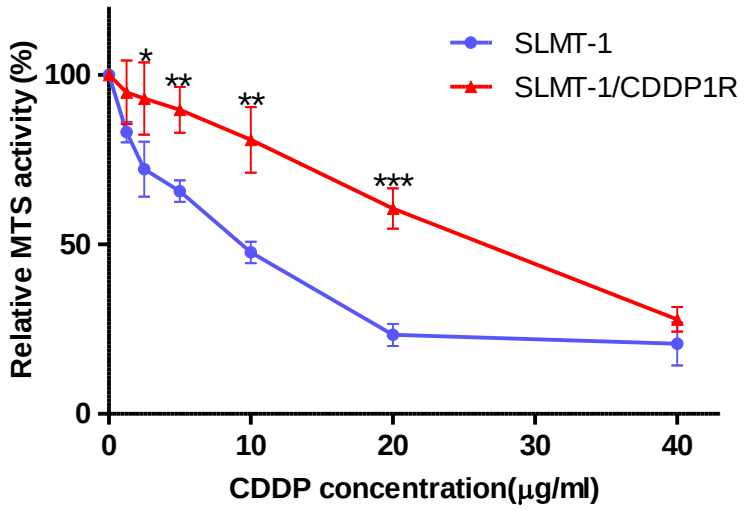


Figure 4.16 Relative MTS activity of SLMT-1 and SLMT-1/CDDP1R cells after 48-hour treatment with cisplatin at different concentrations (0, 1.25, 2.5, 5, 10, 20 and 40μg/mL). *p<0.05 , p<0.01 and *** p<0.001**

Cells of SLMT-1/CDDP1R acquired significant resistance to cisplatin as cells of SLMT-1 showed MTS_{50} (concentration of tested compounds that have 50% inhibition on MTS activity) at 9.1μg/mL while cells of SLMT-1/CDDP1R showed MTS_{50} at 25.8μg/mL (Figure 4.17). SLMT-1/CDDP1R was 2.8-fold more resistant to cisplatin than SLMT-1.\

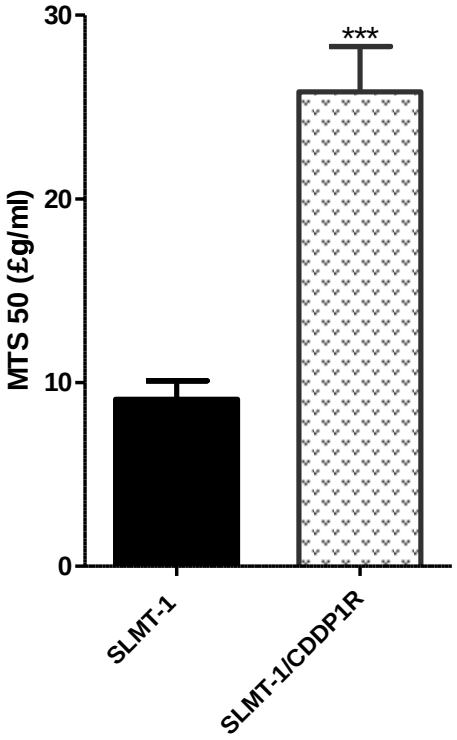


Figure 4.17 MTS_{50} of SLMT-1 and SLMT-1/CDDP1R after 48-hour treatment with cisplatin. *** p<0.001

4.3.1.2. Morphology of Cells

Morphological changes of SLMT-1 cells and SLMT-1/CDDP1R cells cultured in medium containing 9.1μg/mL cisplatin were recorded with digital camera under microscope. As shown in Figure 4.18, SLMT-1 cells shrank and became rounded-up after 48 hours. Increased fragmentation was observed over the time points. For SLMT-1/CDDP1R cells, the confluence increased from the beginning to 72-hour. No reduction in cell volume or size and fragmentation of cells were observed at 48-hour. Increased fragmented cells were found at 72-hour.

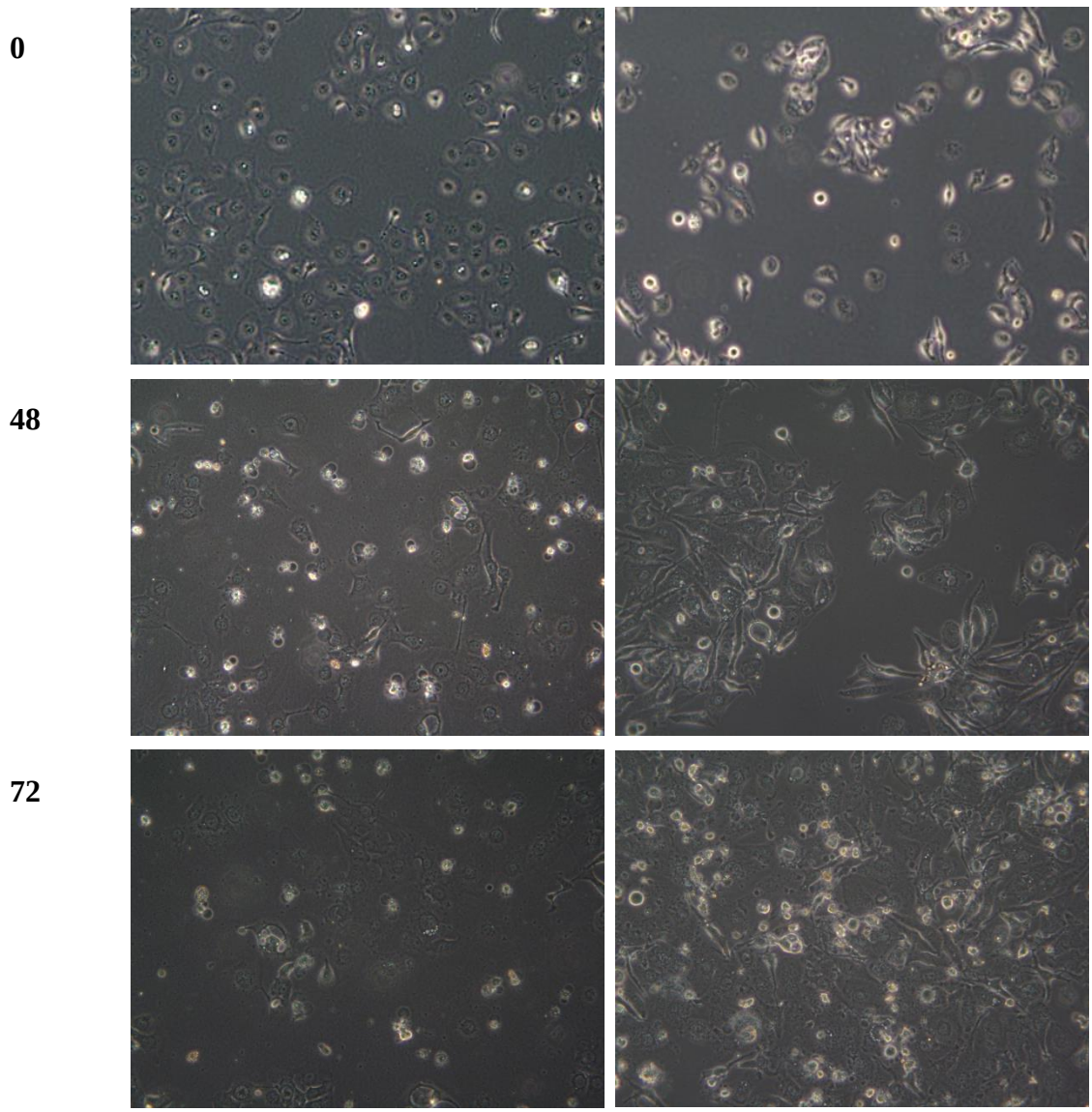


Figure 4.18 Morphology of SLMT-1 cells and SLMT-1/CDDP1R cells after culturing in medium with 9.1μg/mL cisplatin for 0, 48 and 72 hours.

4.3.1.3. Proliferation curve

MTS assay was performed to show the proliferation curve of SLMT-1 and SLMT-1/CDDP1R cultured in medium with or without 9.1μg/mL cisplatin. The proliferation curves are showed in Figure 4.19. Except at the time point of 96-hour, SLMT-1 and SLMT-1/CDDP1R generally showed similar growth rate in culture condition without cisplatin. When the cells were cultured in medium containing 9.1μg/mL cisplatin, SLMT-1/CDDP1R cells grew relatively slower than those in condition with cisplatin whereas the growth of SLMT-1 cells was suppressed. Significant difference in relative growth was observed at time points of 48, 72 and 96-hour for SLMT-1 and SLMT-1/CDDP1R in culturing condition with 9.1μg/mL cisplatin.

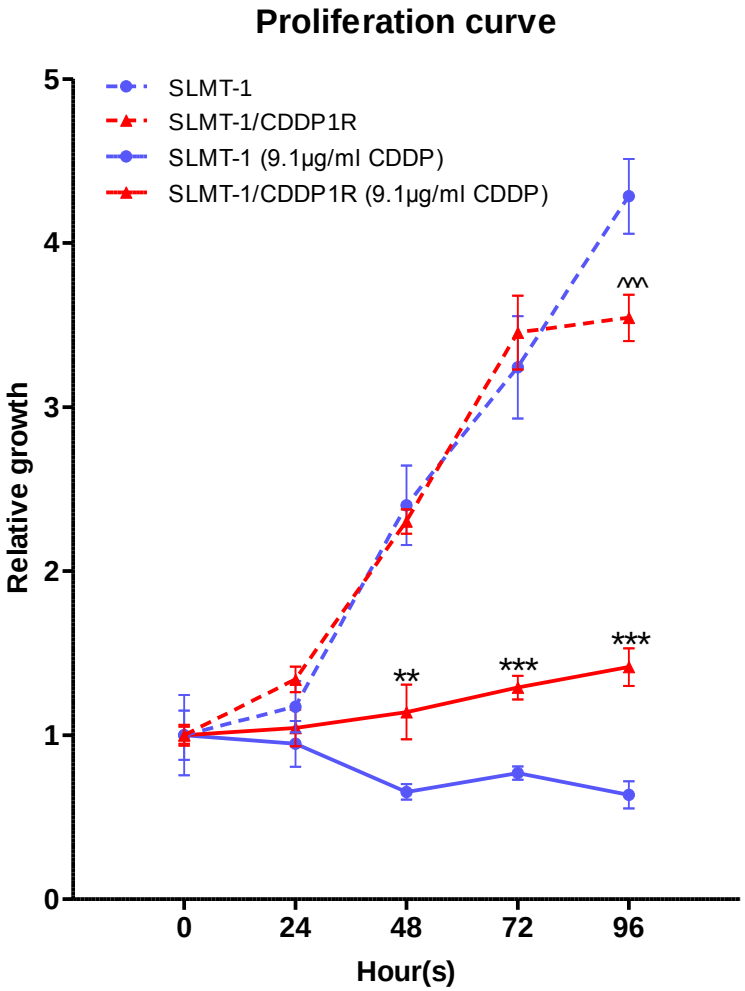


Figure 4.19 Proliferation curves of SLMT-1 and SLMT-1/CDDP1R cultured in medium with or without 9.1μg/mL cisplatin.
Compared to SLMT-1(9.1μg/mL CDDP), ** p<0.01 and *** p<0.001;
Compared to SLMT-1, ^^^ p<0.001.

4.3.2. cDNA microarray analysis

To find out the potential target genes for reversing cisplatin-resistance in SLMT-1, cDNA microarray analysis was conducted to compare the gene expression in SLMT-1 cells and SLMT-1/CDDP1R cells. The fold change of signal intensity of each gene was calculated. The five most down-regulated genes were *IGFBP5*, HSPA6, HLA-DQA2, PE and CDNA FLJ45742fis (Table 4.6). The five most up-regulated genes were LINC00520, SLITRK6, LOC100506377,COL15A1 and TCN1 (Table 4.7).

IGFBP5, which showed the highest fold-change, was selected as the target gene for this study.

Table 4.6 List of the five most down-regulated genes in SLMT-1/CDDP1R cells compared with SLMT-1 cells.

Probe set ID	Gene Title	Signal Intensity		Fold-change
		SLMT-1	SLMT-1/CDDP1R	$(\frac{\text{SLMT}-1}{\text{SLMT}-1/\text{CDDP1R}})$
211959_at	**IGFBP5**, insulin-like growth factor binding protein 5	546.29645	12.5653925	43.476273
213418_at	**HSPA6**, heat shock 70kDa protein 6 (HSP70B')	1227.5509	36.81169	33.346767
212671_s_at	**HLA-DQA2**, major histocompatibility complex, class II, DQ alpha 2 (multiple annotations exist)	655.461	29.2004842	22.446648
201117_s_at	**PE**, carboxypeptidase E	321.73504	19.587101	16.426067
236297_at	**CDNA FLJ45742 fis**, clone KIDNE2016327	147.76297	10.4866295	14.090607

Table 4.7 List of the five most up-regulated genes in SLMT-1/CDDP1R cells compared with SLMT-1 cells.

Probe set ID	Gene Title	Signal Intensity		Fold-change
		SLMT-1	SLMT-1/CDDP1R	$(\frac{\text{SLMT}-1}{\text{SLMT}-1/\text{CDDP1R}})$
1555786_s_at	**LINC00520**, long intergenic non-protein coding RNA 520	59.854332	1010.7408	16.886679
232481_s_at	**SLITRK6**, SLIT and NTRK-like family, member 6	15.702985	215.86028	13.74645
242005_at	**LOC100506377**, uncharacterized LOC100506377	21.538492	191.83107	8.906429
203477_at	**COL15A1**, collagen, type XV, alpha 1	7.45289	65.518486	8.791018
205513_at	**TCN1**, transcobalamin I	77.5835	662.4973	8.539155

4.3.3. Validation of cDNA microarray analysis result

qPCR analysis was performed to validate the downregulation of *IGFBP5* in SLMT-1/CDDP1R. As shown in Figure 4.20, the relative expression level of *IGFBP5* of SLMT-1/CDDP1R cells was significantly lower than that of SLMT-1 cells.

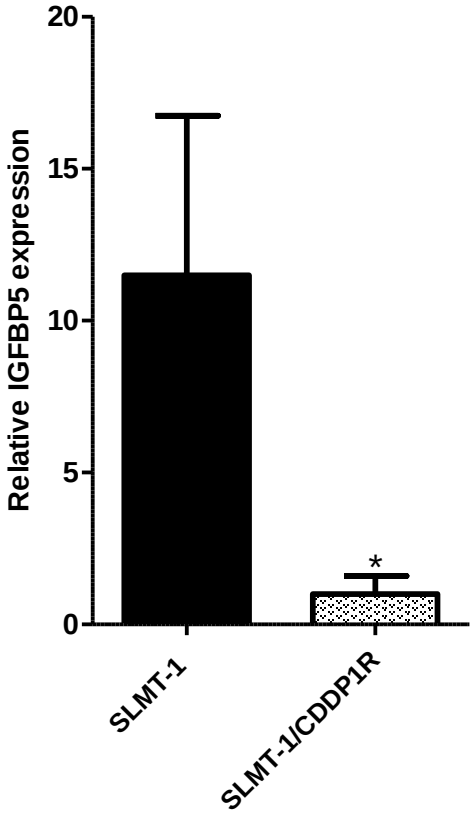


Figure 4.20 Relative expression level of *IGFBP5* in SLMT-1 and SLMT-1/CDDP1R.

Relative *IGFBP5* expressions were determined by comparing with cells of SLMT-1, after normalized with expression of β-actin. * $p<0.05$

4.3.4. Correlation of IGFBP-5 downregulation with acquisition of cisplatin-resistance

Previous results characterized that SLMT-1 cells with cisplatin-resistance showed significant downregulation of *IGFBP5*. The role of *IGFBP5* expression was further revealed in this section of study.

4.3.4.1. Expression level of IGFBP-5 in siRNA-transfected SLMT-1

To further study the role of *IGFBP5* in acquiring cisplatin-resistance, expression of *IGFBP5* was suppressed by siRNA-based RNA interference. siRNA targeting *IGFBP5* was incubated with SLMT-1 cells for 48-hour-transfection. qPCR analysis showed that the expression level of *IGFBP5* was significantly reduced by siRNA-based interference (Figure 4.21).

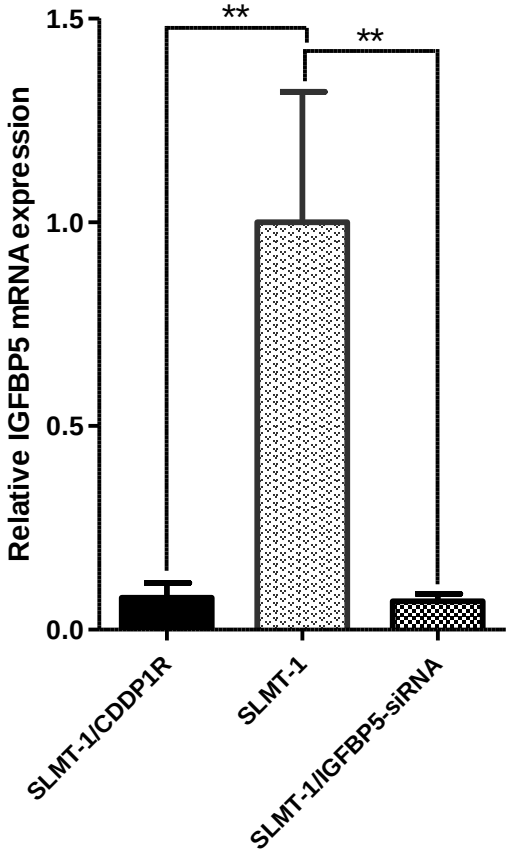


Figure 4.21 Relative expression levels of *IGFBP5* in SLMT-1/IGFBP5-siRNA cells, SLMT-1/CDDP1R and SLMT-1 cells.

Relative *IGFBP5* expression levels were determined by comparing with SLMT-1 cells, after normalized with expression of β-actin. ** $p<0.01$

4.3.4.2. MTS cytotoxicity assay

Sensitivity of SLMT-1/IGFBP5-siRNA cells to cisplatin was assessed by MTS cytotoxicity assay. SLMT-1 transfected with *IGFBP5* targeted siRNA showed significantly higher relative MTS activity than SLMT-1 after 48-hour treatment with cisplatin at concentration of 5-40μg/mL. MTS_{50} of SLMT-1/IGFBP5-siRNA is 20.5μg/mL, which is over 2.3-fold increase in resistance to cisplatin compared with parental SLMT-1. And the increase in cisplatin-resistance in SLMT-1/IGFBP5-siRNA was comparable to that of SLMT-1/CDDP1R (with MTS_{50}=25.8μg/mL).

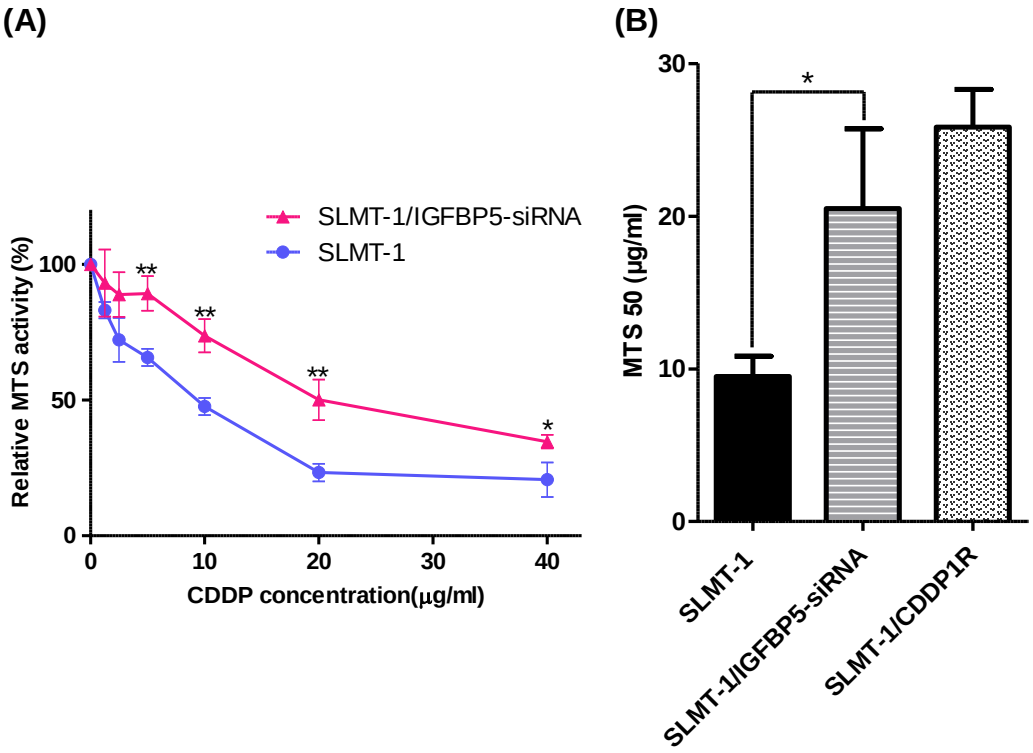


Figure 4.22 (A)Relative MTS activities of SLMT-1,SLMT-1/IGFBP5-siRNA cells after 48-hour treatment with cisplatin at different concentrations (0, 1.25, 2.5, 5, 10, 20and 40μg/mL). (B) MTS_{50} of SLMT-1, SLMT-1/IGFBP5-siRNA after 48-hour treatment with cisplatin. * p<0.05 and ** p<0.01

4.3.5. Reversal of cisplatin-resistance phenotype with *IGFBP5* overexpression

4.3.5.1. Transfection efficiency by immunostaining

Myc-tagged IGFBP5/pcMV3-C-Myc vector and Myc-tagged pcMv/hygro-negative control vector were transfected into SLMT-1 and SLMT-1/CDDP1R cells to give four cell lines: SLMT-1-IGFBP5, SLMT-1-pcMV3, SLMT-1-R-IGFBP5 and SLMT-1-R-pcMV3. The transfection efficiency was evaluated by immunostaining using mouse anti-Myc tag antibody after two months of transfected cells selection. As shown in Figure 4.23, cells of SLMT-1-IGFBP5, SLMT-1-pcMV3, SLMT-1-R-IGFBP5 and SLMT-1-R-pcMV got nuclei in positive staining (dark brown signals with more than 50% of cells). While cells of SLMT-1 and SLMT-1/CDDP1R which were not transfected with Myc-tagged vector got nuclei with negative staining (blue). This reflected the effective transfection of Myc-tagged IGFBP5/pcMV3-C-Myc vector and Myc-tagged pcMv/hygro-negative control vector in all the four transfected cell lines.

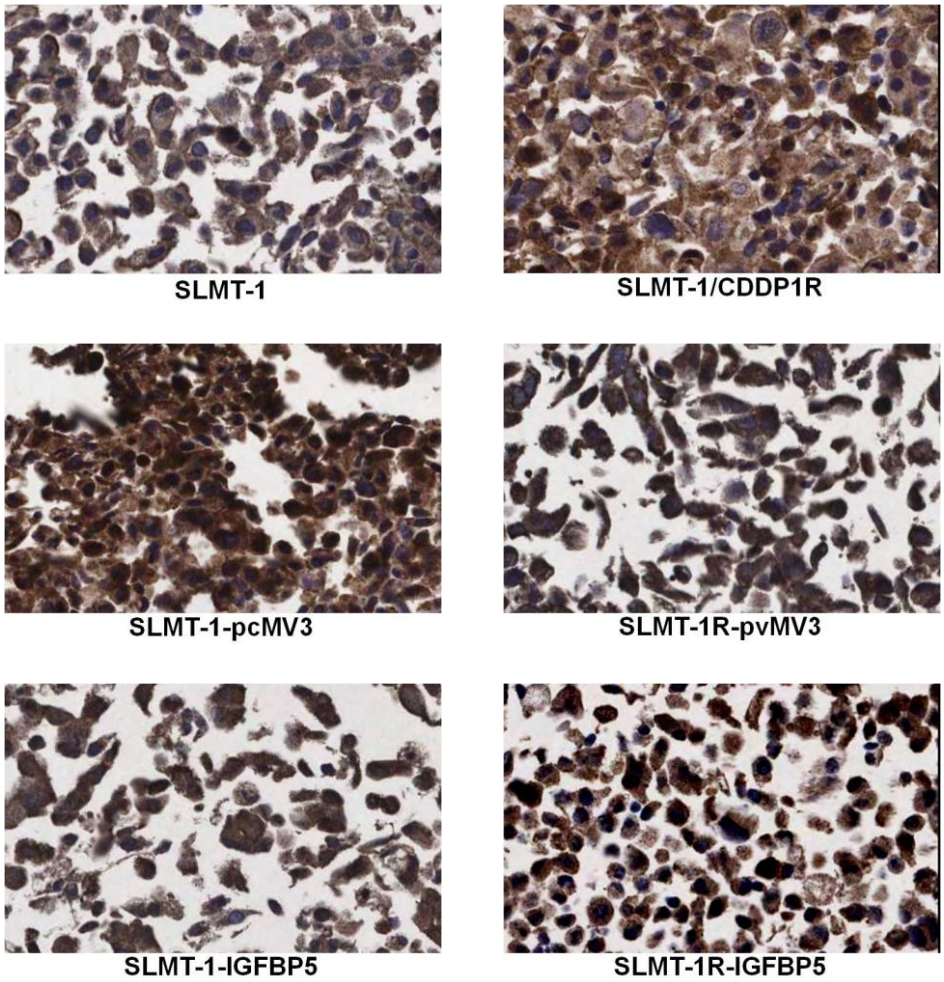


Figure 4.23 Immunostaining of Myc in SLMT-1, SLMT-1/CDDP1R, SLMT-1-pcMV3, SLMT-1-R-pcMV3, SLMT-1-IGFBP5 and SLMT-1-R-IGFBP5. (original magnification×400)

4.3.5.2. Expression of *IGFBP5* in transfected cells

The *IGFBP5* expression levels of transfected cell lines were evaluated by qPCR analysis. As shown in Figure 4.24, SLMT-1-IGFBP5 cells and SLMT-1-pcMV3 cells showed no significant difference (p= 0.4590 & p=0.7411) compared with SLMT-1 parental cell. On the other hand, transfection of IGFBP5/pcMV3-C-Myc vector into SLMT-1/CDDP1R cells was found remarkably enhancing the expression of *IGFBP-5* in SLMT-1R-IGFBP5. The transfection of pcMv/hygro-negative control vector into SLMT-1/CDDP1R cells did not influence the expression of *IGFBP-5* (p=0.4790).

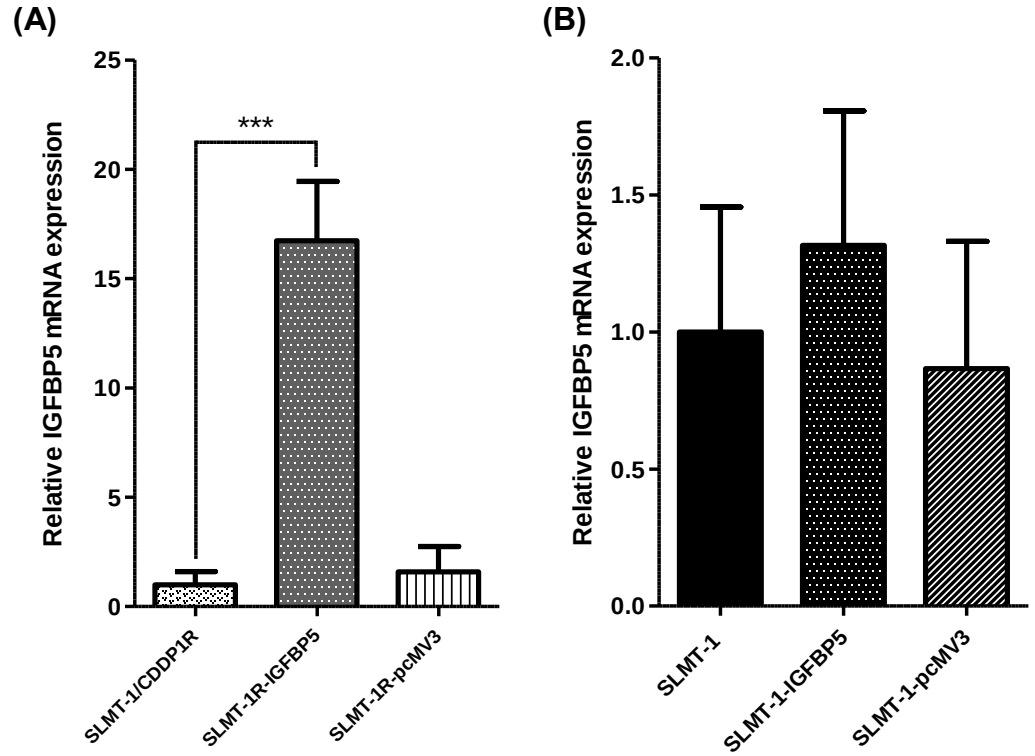


Figure 4.24 Relative expression levels of *IGFBP5* in (A) SLMT-1R-IGFBP5 and SLMT-1R-pcMV3 compared with SLMT-1/CDDP1R;
(B) SLMT-1-IGFBP5 and SLMT-1-pcMV3 compared with SLMT-1, after normalized with expression of β-actin. *** p<0.001

4.3.5.3. MTS cytotoxicity assay

MTS assay was performed to compare the sensitivity of SLMT-1R-IGFBP5 cells to cisplatin with that of SLMT-1/CDDP1R cells and SLMT-1 cells. SLMT-1R-pcMV3, SLMT-1-IGFBP5 and SLMT-1-pcMV3 cells were examined as control cell lines.

Relative MTS activity of all six cell lines at different concentration of cisplatin was shown in Figure 4.25.

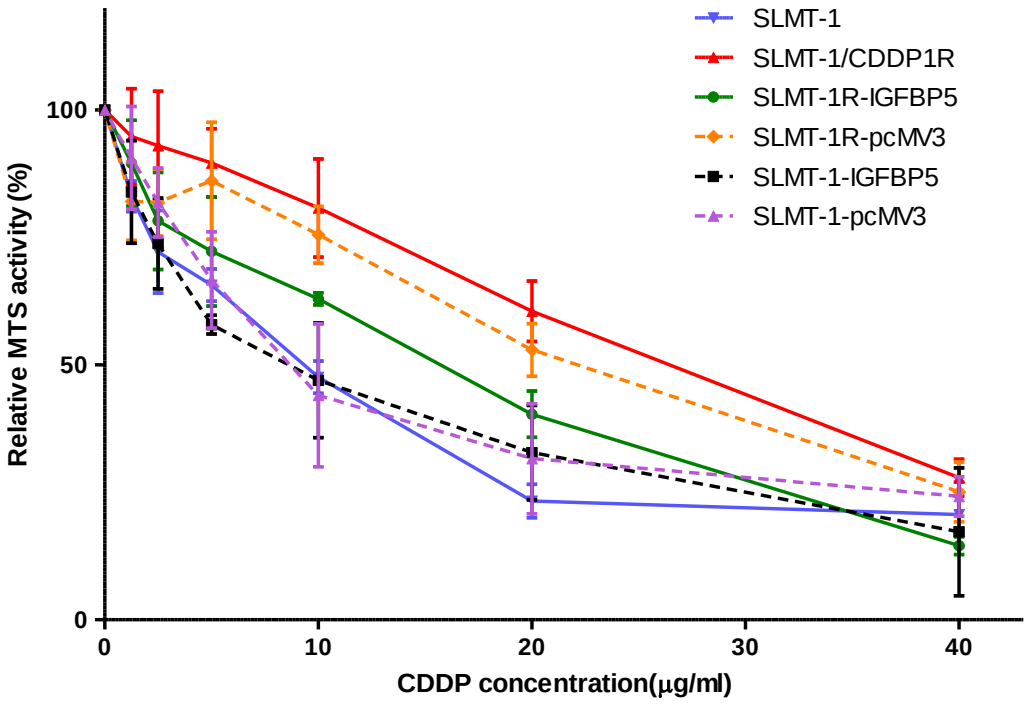


Figure 4.25 Relative MTS activity of SLMT-1, SLMT-1/CDDP1R, SLMT-1R-IGFBP5, SLMT-1R-pcMV3, SLMT-1-IGFBP5 and SLMT-1-pcMV3 cells after 48-hour treatment with cisplatin at different concentrations (0, 1.25, 2.5, 5, 10, 20and 40μg/mL).

MTS_{50} of all six cell lines were shown in Figure 4.26, SLMT-1R-IGFBP5 showed MTS_{50} at 15.3μg/mL, which was significantly lower than that of SLMT-1/CDDP1R (25.8μg/mL). Upregulation of *IGFBP5* by IGFBP5/pcMV3-C-Myc vector in SLMT-1/CDDP1R was able to reduce cisplatin-resistance by 41%. For the control cell lines, MTS_{50} of SLMT-1R-pcMV3 was found to be 21.8μg/mL which was considered as no significant difference (p=0.4790) with that of SLMT-1/CDDP1R. This indicated that expression of pcMV3 did not contribute to the reduction in MTS_{50} in SLMT-1R-IGFBP5. MTS_{50} of SLMT-1-pcMV3 and SLMT-1-IGFBP5 were 9.7μg/mL and 9.6μg/mL respectively, which showed no significant differences (p=0.4590 and p=0.7411) with MTS_{50} of SLMT-1 (9.1μg/mL). These implied that expression of *IGFBP5* and mock vector in parental SLMT-1 did not affect sensitivity of cisplatin-sensitive SLMT-1 to cisplatin.

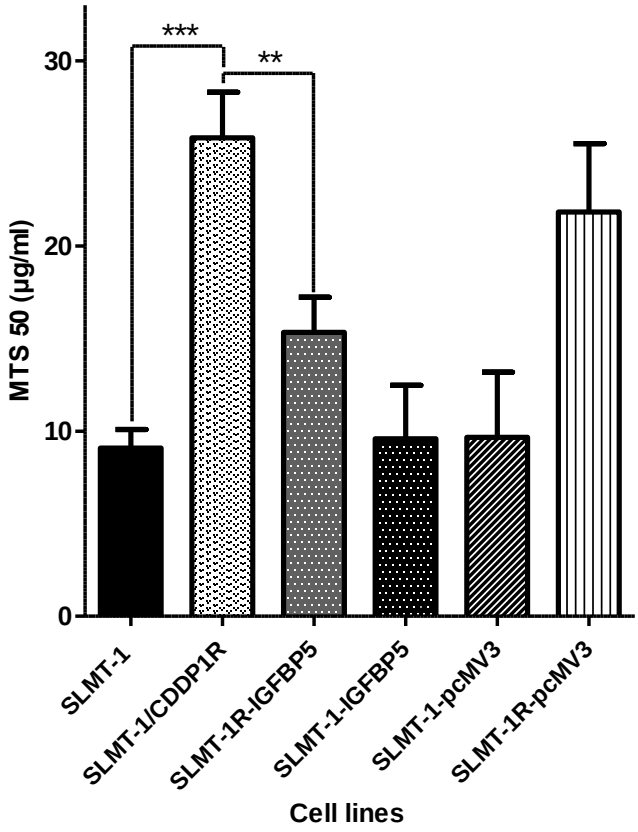


Figure 4.26 MTS_{50} of SLMT-1, SLMT-1/CDDDP1R, SLMT-1R-IGFBP5, SLMT-1-IGFBP5, SLMT-1-pcMV3 and SLMT-1R-pcMV3 after 48-hour treatment with cisplatin. ** p<0.01, *** p<0.001

4.3.5.4. Proliferation curve

MTS assay was performed to show the proliferation curve of SLMT-1R-IGFBP5 cultured in medium with or without 9.1μg/mL cisplatin (MTS_{50} value of cisplatin to treat SLMT-1). The proliferation curves were showed in Figure 4.27, SLMT-1R-IGFBP5 and SLMT-1/CDDP1R generally showed similar growth rate in culture condition without cisplatin except at the time point of 72-hour. When the cells were cultured in medium containing 9.1μg/mL cisplatin, the proliferation of SLMT-1R-IGFBP5 cells was suppressed. Significant differences were found in relative growth at time points of 48, 72 and 96-hour, meaning that SLMT-1R-IGFBP5 is more CDDP-sensitive than SLMT-1/CDDP1R at these time points.

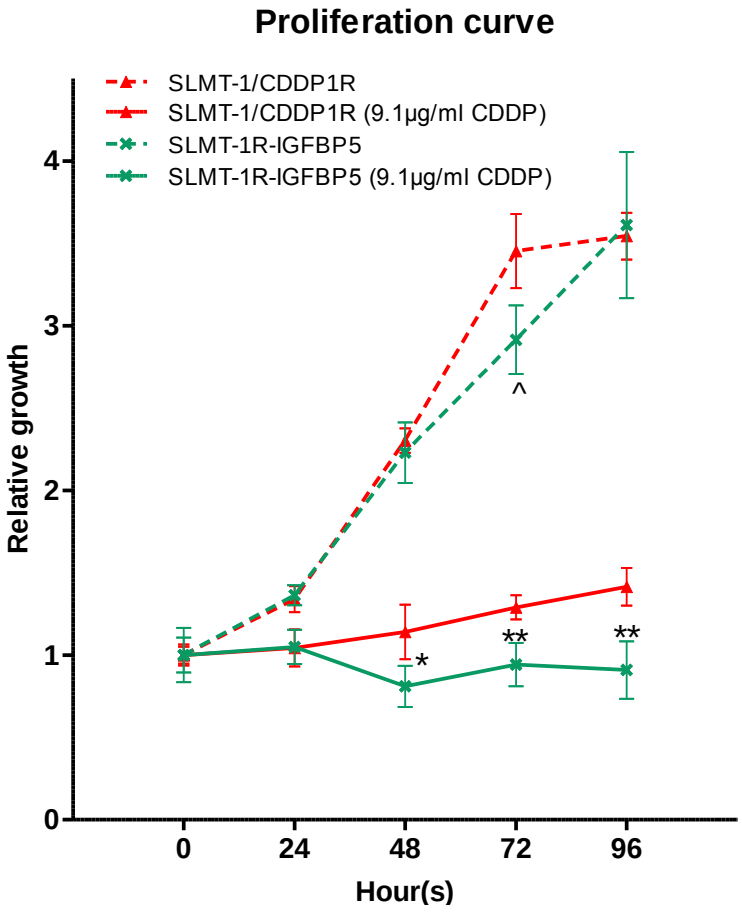


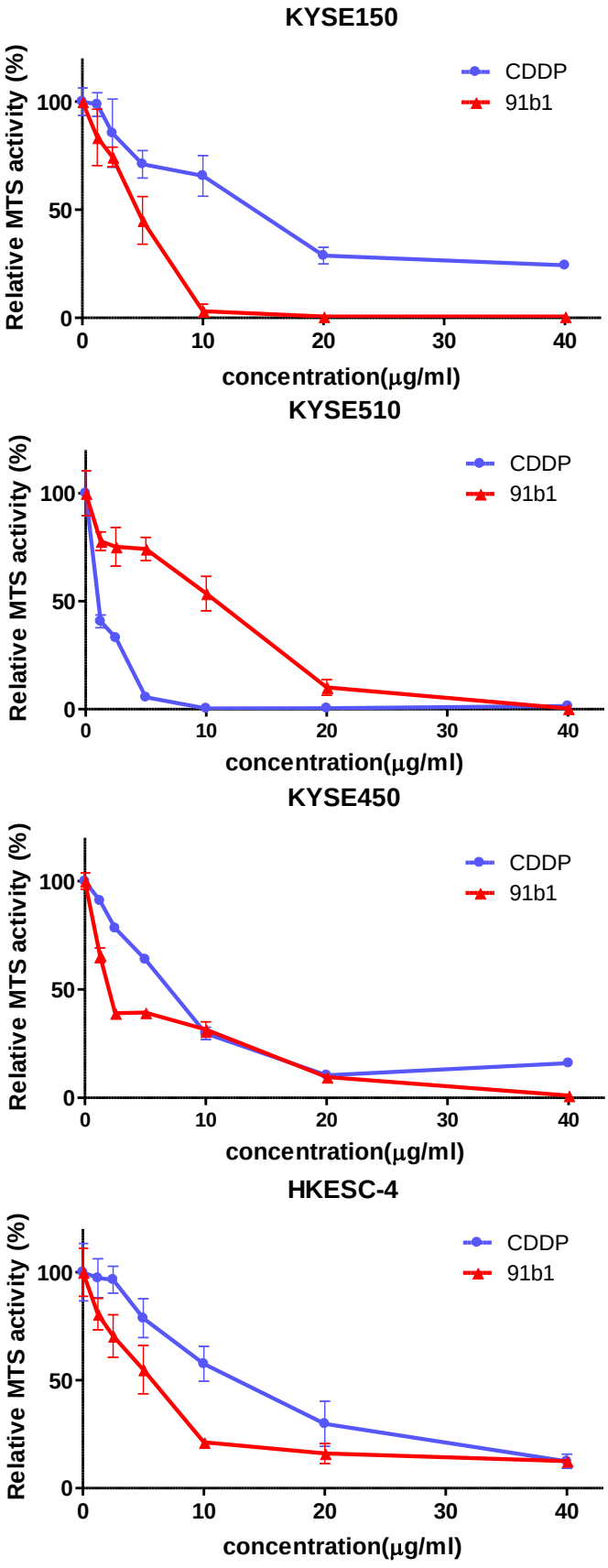
Figure 4.27 Proliferation curves of SLMT-1R-IGFBP5 and SLMT-1/CDDP1R cultured in medium with or without 9.1μg/mL cisplatin. Compared to SLMT-1/CDDP1R (9.1μg/mL CDDP), * $p < 0.05$ and ** $p<0.01$; Compared to SLMT-1/CDDP1R, ^ $p<0.05$

4.4. Study of anti-cancer effect of 91b1 on ESCC cells

4.4.1. Cytotoxic effect of 91b1 on ESCC cells

In order to examine the anti-cancer effect of 91b1 on ESCC and non-tumor cell lines, the percentage of MTS activity was plotted against concentration of 91b1 and CDDP. CDDP was applied as the positive control (Figure 4.28).

The plots of percentage of MTS activity showed that 91b1 exerted cytotoxic effect in all seven cell lines in a dose dependent manner as CDDP did but in different extent.


KYSE150
CDDP
91b1
Relative MTS activity (%)
100
50
0
0
10
20
30
40
concentration(μg/ml)
KYSE510
CDDP
91b1
Relative MTS activity (%)
100
50
0
0
10
20
30
40
concentration(μg/ml)
KYSE450
CDDP
91b1
Relative MTS activity (%)
100
50
0
0
10
20
30
40
concentration(μg/ml)
HKESC-4
CDDP
91b1
Relative MTS activity (%)
100
50
0
0
10
20
30
40
concentration(μg/ml)

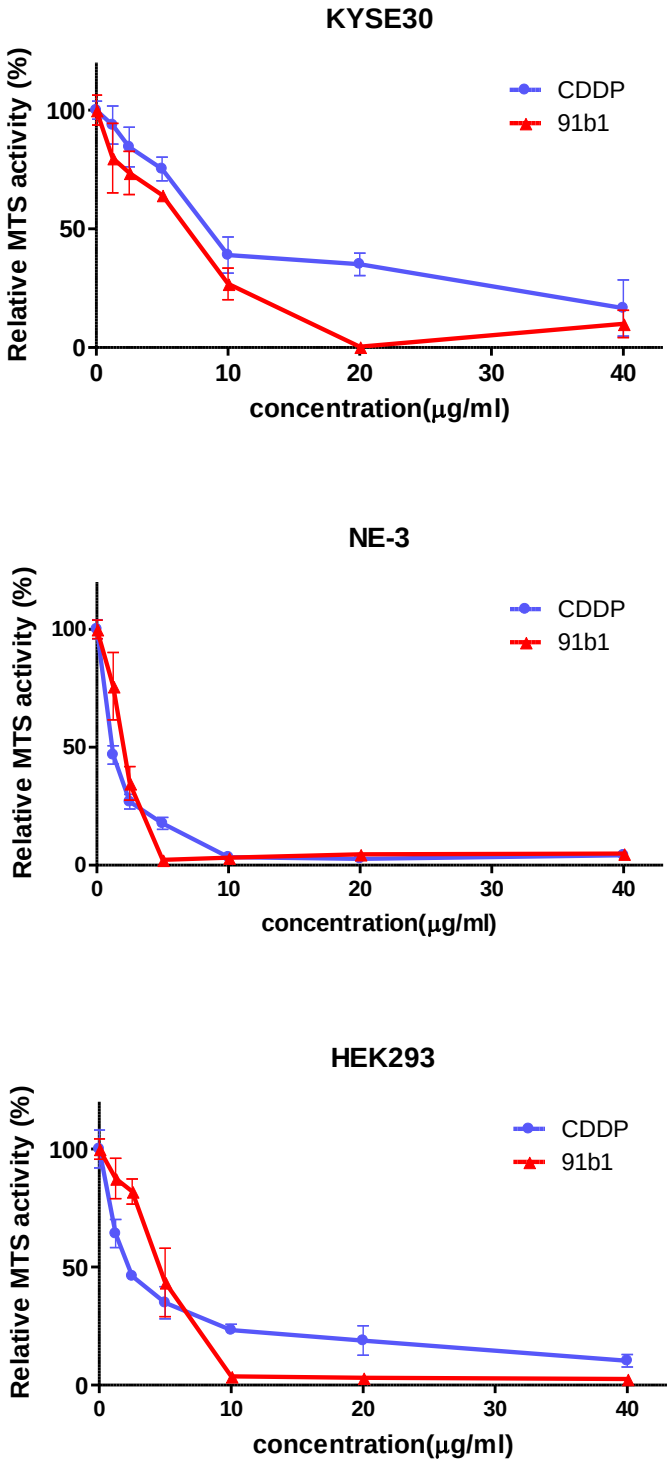


Figure 4.28 Cytotoxic effect of 91b1 on ESCC cells (KYSE150, KYSE510, KYSE450, HKESC-4 and KYSE30) and non-tumor cells (NE-3 & HEK293). CDDP was used as the positive control.

The cytotoxic effects of 91b1 on ESCC cell line and non-tumor cells lines were compared with CDDP, MTS_{50} values (concentration of tested compounds that have 50% inhibition on MTS activity) were determined from three independent experiments and were summarized in Table 4.8.

Table 4.8 MTS_{50} (μg/mL) of 91b1 and CDDP for five ESCC cell lines and two non-tumor cell lines. Results were expressed with mean± SD from triplicate experiments.

Cell lines	MTS_{50}	
	91b1(μg/mL)	CDDP(μg/mL)
KYSE150	4.55±0.77	13.16±2.54
KYSE450	1.80±0.23	6.69±0.34
KYSE510	10.23±1.75	0.95±0.10
HKESC-4	4.75±1.83	11.88±1.52
KYSE30	6.50±0.41	8.00±1.08
NE-3	1.94±0.29	1.18±0.21
HEK293	4.55±0.87	2.19±0.25

MTS_{50} values of 91b1 were lower than those of CDDP in four ESCC cell lines (KYSE150, KYSE450, HKESC-4 and KYSE30), implying that 91b1 showed stronger anti-cancer effect than CDDP in these ESCC cell lines. MTS_{50} values of CDDP for non-tumor cell lines, NE-3 and HEK293, were 1.18μg/mL and 2.19μg/mL respectively, and those for 91b1 on NE-3 and HEK293 were 1.94μg/mL and 4.55μg/mL respectively. This indicated that 91b1 may be less toxic to non-tumor cells than CDDP.

4.4.2. cDNA microarray analysis

To examine the changes in gene expression after 48-hour-treatment with 91b1, cDNA microarray analysis was conducted to compare the gene expression in 91b1-treated KYSE150 cells (9.5μg/mL 91b1 for 48 hours) and control KYSE150 cells (treated with vehicle DMSO for 48hour). The dose of 9.5μg/mL was used due to 70% cytotoxicity effect induced on the KYSE150 cells. The fold changes of normalized signal intensity of each gene were calculated. The five most downregulated genes were *CCL5*, *LUM*, *STON1*, *IGFBP5* and *CP* while the five most upregulated genes were *C7orf57*, *ZBED2*, *CLGN*, *CSF2* and *SLC16A6*. (Table 4.9 & 4.10)

Change in expression of CCL5, chemokine(C-Cmotif) ligand 5, the one with highest fold change (downregulaed by 2.11 times; Table 4.9) in microarray analysis was verified by qPCR analysis in section 4.4.3.

Table 4.9 List of the five most down-regulated genes in KYSE150 cells treated with 9.5μg/mL 91b1 for 48 hours compared with control cells.

Probe set ID	Gene Title	Signal Intensity		Fold-change
		91b1 treated	control	$(\frac{\text{control}}{\text{91b1-treated}})$
1405_i_at	**CCL5**, chemokine(C-Cmotif) ligand 5	3.734910	7.911000	2.118123
201744_s_at	**LUM**,lumican	3.719280	7.694374	2.068781
213413_at	**STON1**,stonin 1	4.321197	7.786885	1.802020
211959_at	***IGFBP5***, insulin-like growth factor binding protein 5	3.443796	6.158643	1.788330
1558034_s_at	**CP**, ceruloplasmin	3.963314	7.085329	1.787728

Table 4.10 List of the five most up-regulated genes in KYSE150 cells treated with 9.5μg/mL 91b1 for 48 hours compared with control cells.

Probe set ID	Gene Title	Signal Intensity		Fold-change
		91b1 treated	control	$(\frac{\text{control}}{\text{91b1-treated}})$
1557636_a_at	**C7orf57**,chromosome 7 open reading frame 57	5.131972	2.715359	1.889979
219836_at	**ZBED2**, zinc finger, BED-type containing 2	10.007047	5.850765	1.710383
205830_at	**CLGN**, calmegin	4.864191	2.923666	1.66373
210229_s_at	**CSF2**, colony stimulating factor 2	7.673194	4.657937	1.647337
230748_at	**SLC16A6**, solute carrier family 16, member 6	6.679981	4.237427	1.576424

4.4.3. Validation of cDNA microarray result

To validate the downregulation of *CCL5* after 91b1-treatment in cDNA microarray analysis. qPCR analysis was conducted using primer specific for *CCL5* and β-actin. KYSE150 showed significant decrease in relative expression level of *CCL5*. The qPCR result was found consistent with the result of cDNA microarray analysis

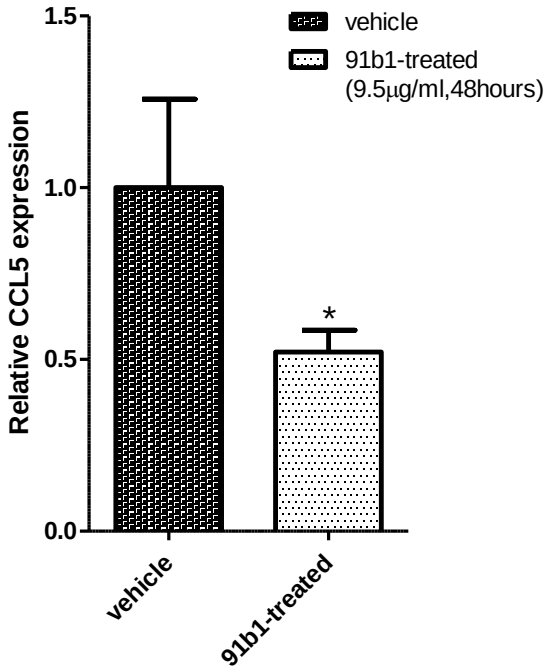


Figure 4.29 Relative *CCL5* expression level after 48-hour treatment with 91b1 and vehicle in KYSE150. The relative CCL5 expression level was determined by comparing with cells treated with vehicle after normalized with expression of β-actin using qPCR. * p<0.05

4.4.4. CCL5 mRNA expression in cell lines by qPCR

mRNA expression levels of *CCL5* of five ESCC cell lines and two non-tumor cell lines were examined by qPCR (Figure 4.30) The relative *CCL5* expression levels were compared with non-tumor esophageal epithelial cell line NE-3 after being normalized with the expression of *β-actin*.

Another non-tumor cell line HEK293, embryonic kidney cell line, showed relatively lower expression level of *CCL5* than NE-3.

Four ESCC cell lines HKESC-4, KYSE450, KYSE510 and KYSE150 showed higher expression level of *CCL5* than NE-3. These four cell lines were used for investigating the cytotoxic effect of 91b1 on ESCC. KYSE30 showed relatively lower expression level of *CCL5* than NE-3 and the others ESCC cell lines, and thus was used in *CCL5* functional study in section 4.4.6.3.

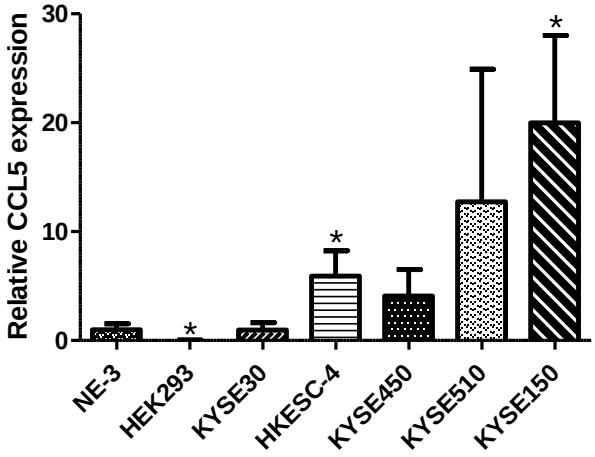


Figure 4.30 Relative mRNA expression levels of *CCL5* in five ESCC cell lines and two non-tumor cell lines. The relative CCL5 mRNA expression level was determined by comparing with NE-3, after being normalized with expression of β-actin. * $p<0.05$

4.4.5. Validation on the expression of CCL5 after 91b1 treatment

4.4.5.1. mRNA expression by qPCR

qPCR analysis was conducted to examine the mRNA expression of *CCL5* in four ESCC cell lines after 48-hour-treatment with 91b1 of different concentrations (6.5, 9.5 & 21μg/mL) (Figure 4.31). Relative *CCL5* expression was determined by comparing the cells treated with DMSO (0.05%) vehicle, after being normalized with expression of β-actin. DMSO, vehicle for 91b1, was incubated with cells for 48 hours before qPCR analysis.

All four ESCC cell lines showed reduction in *CCL5* mRNA expression after treatment with 91b1, which is in-line with the microarray results using KYSE150. In generally, the suppression effect of 91b1 on *CCL5* expression was dose-dependent. These implied CCL5 is one of the downstream targets of cytotoxic effect of 91b1.

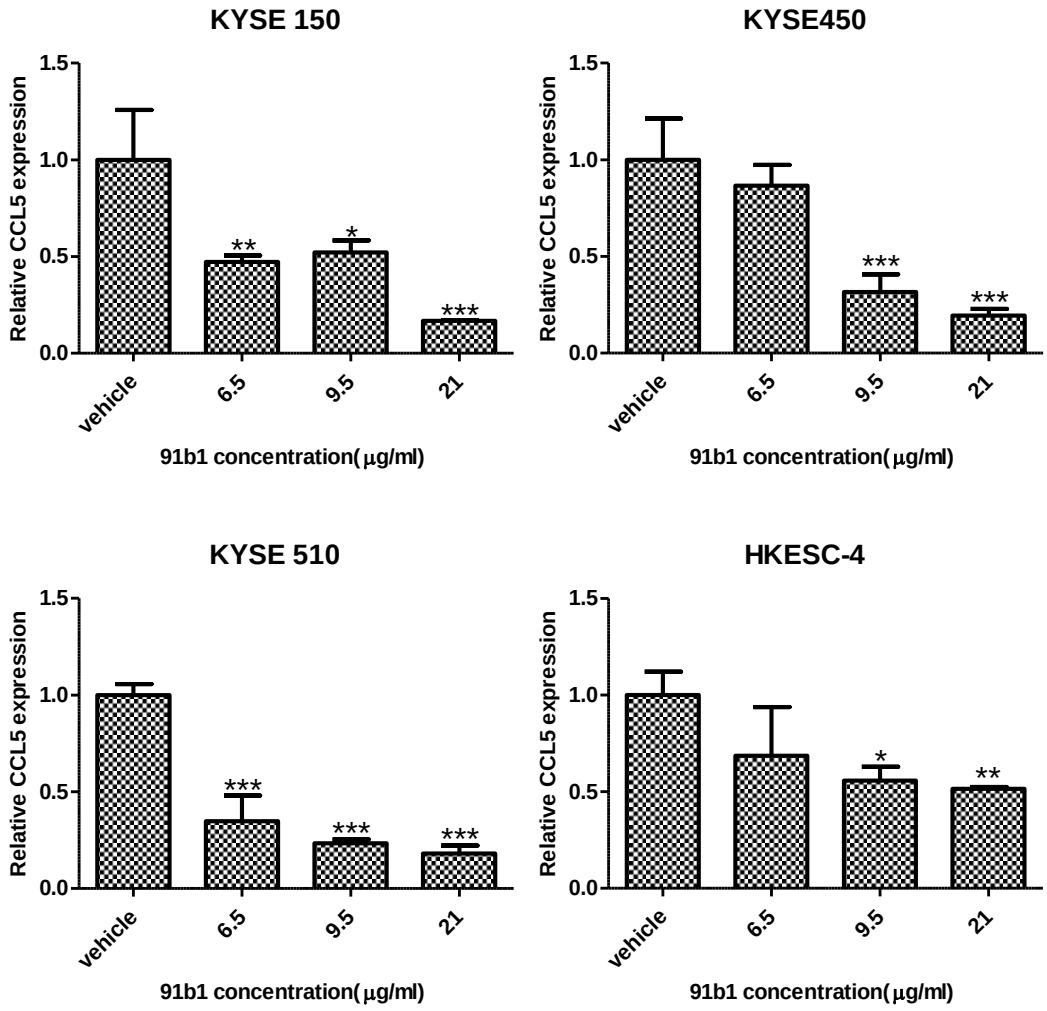


Figure 4.31 *CCL5* mRNA expression levels in ESCC cell lines KYSE150, KYSE450, KYSE510 and HKESC-4 after 48-hour treatment of 91b1 in different concentrations. Relative *CCL5* expression levels were determined by comparing with cells treated with DMSO (0.05%), vehicle for 91b1, after normalized with expression of β-actin. * p<0.05, **p<0.01 and *** p<0.001

4.4.5.2. Protein expression of CCL5 studied by immunostaining

The effect of 91b1 treatment on protein expression of CCL5 was demonstrated by IHC staining with CCL5 antibody. KYSE150 cells were treated with 91b1 in different concentrations or vehicle DMSO control for 48hours. The amount of cells with staining in brown colour revealed the relative protein expression of CCL5. Dark-brownish stained cells in vehicle-control revealed high CCL5 protein expression. Lesser CCL5 positive cells were shown by cells treated with increasing concentration of 91b1 for 48 hours (Figure 4.32), suggesting that the protein expression of CCL5 was reduced when the cells were treated in increasing concentration of 91b1.

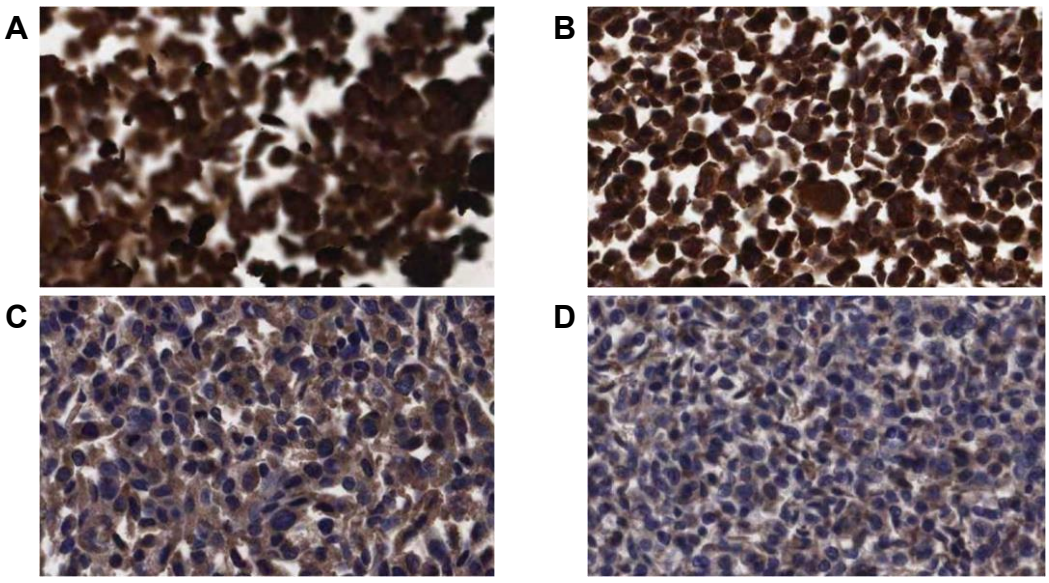


Figure 4.32 Immunostaining results of CCL5 for KYSE150 treated with (A) vehicle DMSO control (B) 6.5µg/mL 91b1 (C) 9.5µg/mL 91b1 and (D) 21µg/mL for 48 hours. (original magnification x400)

4.4.5.3. CCL5 protein expression in cell lines by ELISA

The protein expression of CCL5 was evaluated using ELISA specific for CCL5 protein. As shown in Figure 4.33, KYSE 150, KYSE510 and KYSE450 expressed CCL5 protein in significantly higher level than NE-3, approximately 4 to 37 folds of that of NE-3. For HKESC-4, the protein expression of CCL5 was around 1.7 folds of NE-3.

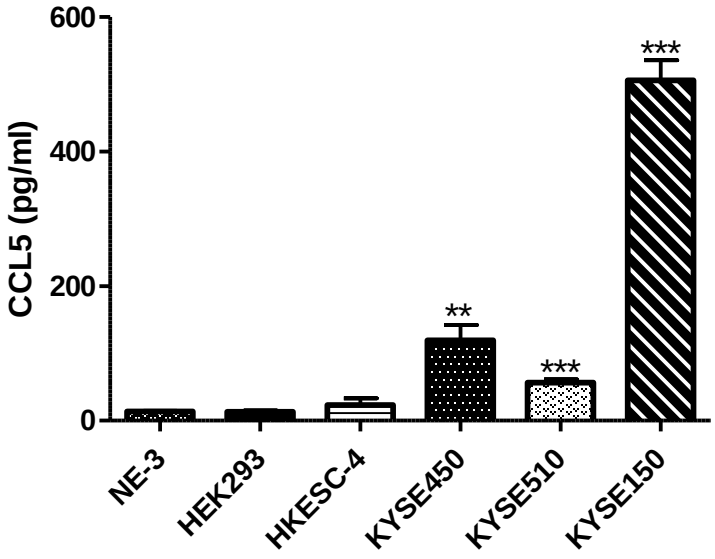


Figure 4.33 CCL5 protein expression level of in four ESCC cell lines and two non-tumor cell lines. ** p<0.01 and* p<0.001.**

Figure 4.34 shows the protein expression of CCL5 in four ESCC cell lines after 48-hour of treatment with 91b1 in concentration of 6.5μg/mL, 9.5μg/mL, 21μg/mL. Cells treated with DMSO (0.05%) for 48 hours were also tested with ELISA analysis as the vehicle control. Reduction in amount of CCL5 protein was observed in both four cell lines. Treatment with increasing concentrations of 91b1 resulted in decreasing in protein level of CCL5.

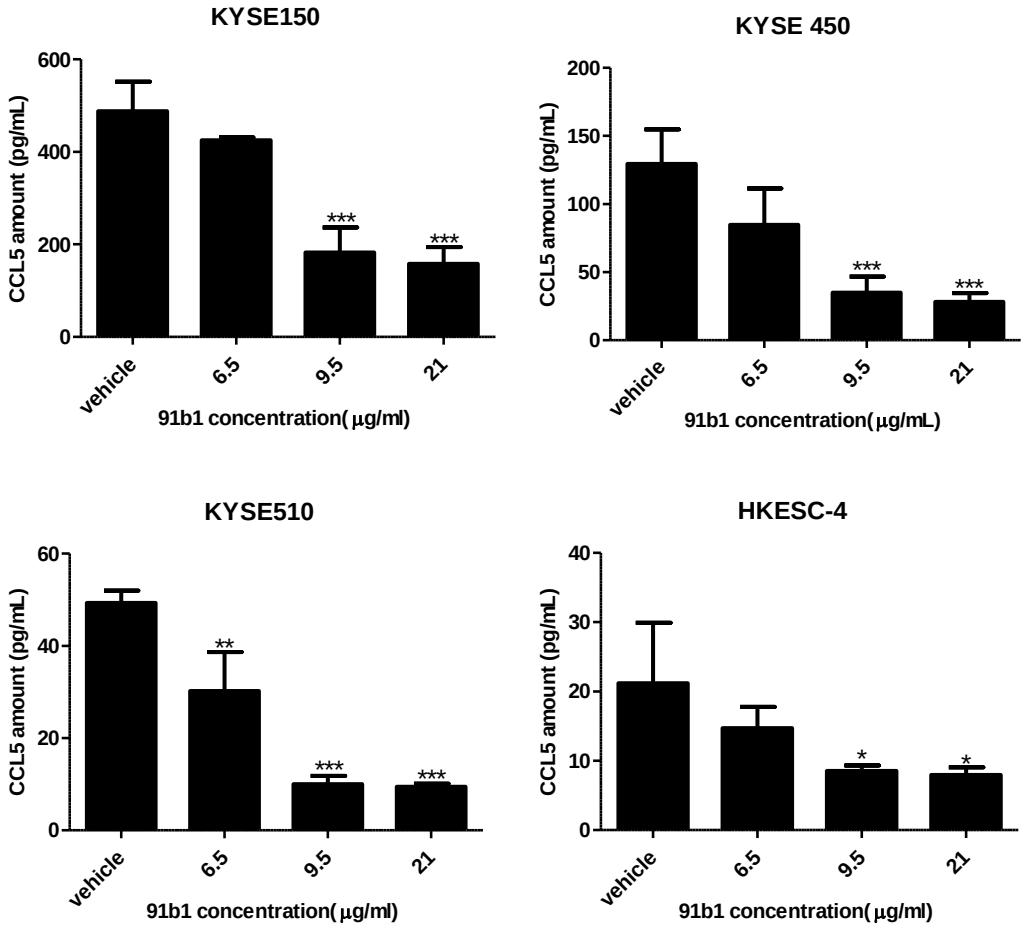


Figure 4.34 CCL5 protein expression of KYSE150, KYSE450, KYSE510 and HKESC-4 after 48-hour treatment of 91b1 in different concentrations Compared with vehicle, * p<0.05, ** p<0.01 and* p<0.001.**

4.4.6. Characterization of CCL5

4.4.6.1. CCL5 protein expression in ESCC cell lines by immunostaining

IHC staining was employed to detect the protein expression level of CCL5 in eight ESCC cell lines and non-tumor cell line NE-3. Compared to NE-3, stronger staining was showed in KYSE150, KYSE510, KYSE450, KYSE520, HKESC-3, HKESC-4 and SLMT-1, implying these ESCC cell lines had relatively higher protein expression of CCL5 than NE-3. Whereas KYSE30 got more unstained cells than NE-3, indicating KYSE30 had relatively lower CCL5 protein expression than NE-3. Up-regulation of CCL5 was detected in 87.5% (7/8) of ESCC cell lines.

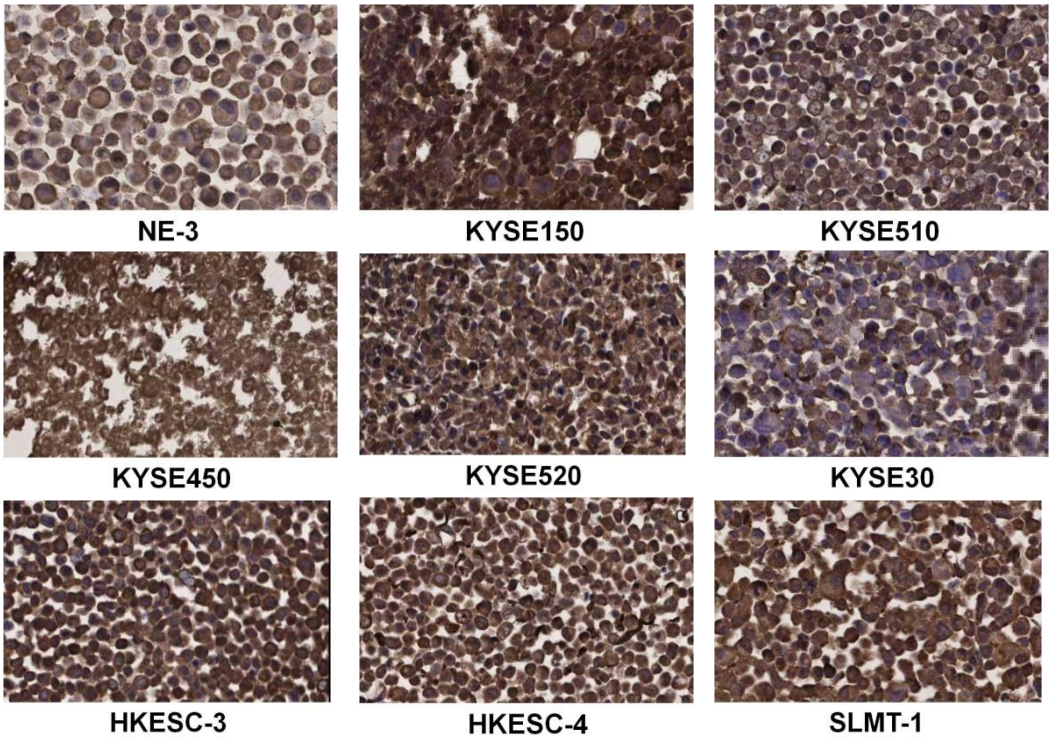


Figure 4.35 Immunostaining of CCL5 in eight ESCC cell lines and non-tumor cell line NE-3. (original magnification×400)

4.4.6.2. CCL5 protein expression in ESCC patient samples

CCL5 expression level was examined by immunohistochemical staining in 26 ESCC tumor tissues, 15 non-tumor tissues and 16 pre-malignant tissues. IHC staining of *CCL5* was observed in cytoplasm of cells. IHC staining showing high expression and low expression of CCL5 were shown in Figure 4.36.

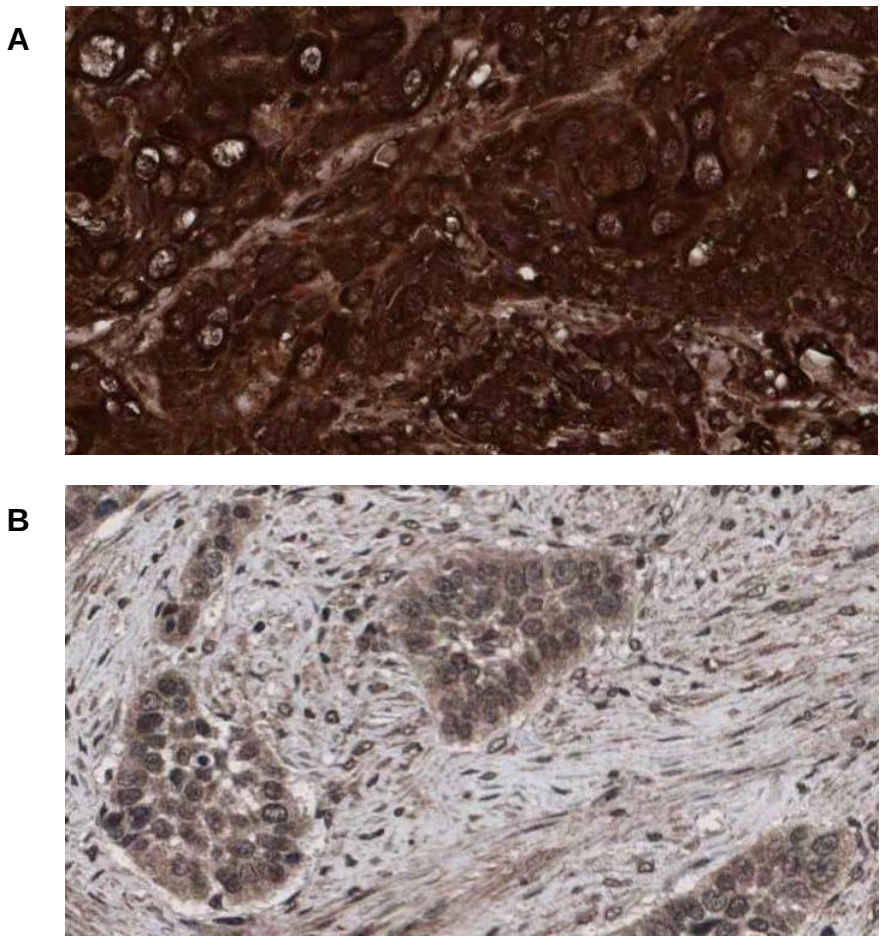


Figure 4.36 Representative photos of immunohistochemical staining of CCL5 in ESCC specimens graded as (A) high expression and (B) low expression (original magnification ×400)

Tissue specimens were classified according to their histopathological features into three categories including tumor tissues, non-tumor tissues and pre-malignant tissues (hyperplasia and dysplasia).

High expression level of CCL5 was observed in 76.9% (20/26) of tumor tissues, 62.5% (10/16) of pre-malignant tissues and only 40.0% (6/15) of non-tumor epithelial esophageal tissues (Table 4.11). Tumor tissues expressed CCL5 protein in high level more frequent than non-tumor epithelial esophageal tissues (p=0.018). Pre-malignant tissues (10/16) expressed CCL5 protein in high level than non-tumor epithelial esophageal tissues. However, statistically significant difference was not observed (p=0.224).

Table 4.11 Summary of results showing the expression of CCL5 in non-tumor, pre-malignant and tumor tissues

Histopathological features	No. of cases	High expression	Low expression
Non-Tumor	15	6 (40.0%)	9 (60.0%)
Pre-malignant tissues	16	10 (62.5%)	6 (37.5%)
Tumor	26	20 (76.9%)	6 (23.1%)

4.4.6.3. Trans-well matrigel invasion assay

The change in cell invasion ability was tested by trans-well matrigel invasion assay. KYSE30 cells were seeded with rhCCL5 in concentrations of 0ng/mL, 50 ng/mL, 100 ng/mL and 500 ng/mL. Transmembrane cells were stained with crystal violet and counted under the light microscope. Invasion percentage was calculated based on number of transmembrane cells in migration assays 24 hours after seeding the cells.

As shown in Figure 4.37A, no transmembrane cells were detected in invasion assay without recombinant human CCL5 protein (rhCCL5) after 24 hours. And increasing number of transmembrane cells were detected with increase in rhCCL5 concentration. The percentage of invasion increased with the concentration of rhCCL5 (Figure 4.38), suggesting that the invasion ability of ESCC cells increased with the concentration of rhCCL5 added.

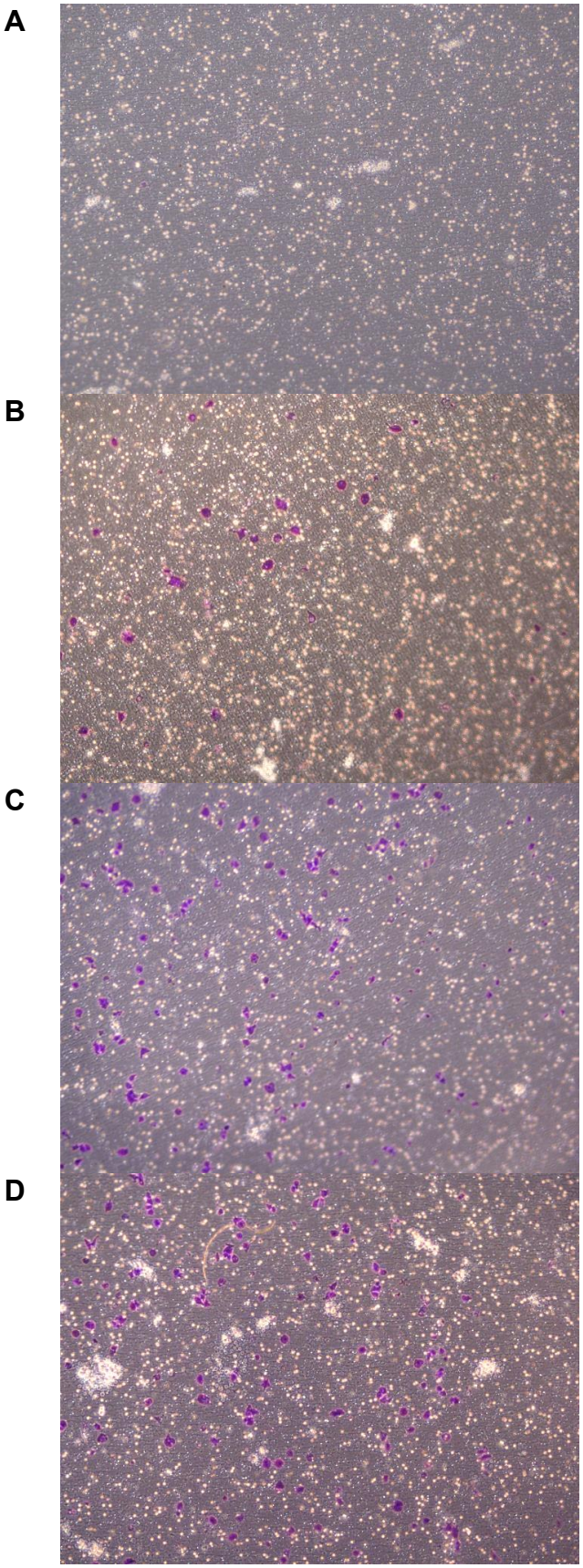


Figure 4.37 Cell Invasion assay by trans-well matrigel chamber (A) 0 ng/ml rhCCL5, (B) 50 ng/ml rhCCL5, (C) 100 ng/ml rhCCL5 and (D) 500 ng/ml rhCCL5. Transmembrane cells were stained by crystal violet. (original magnification ×200)

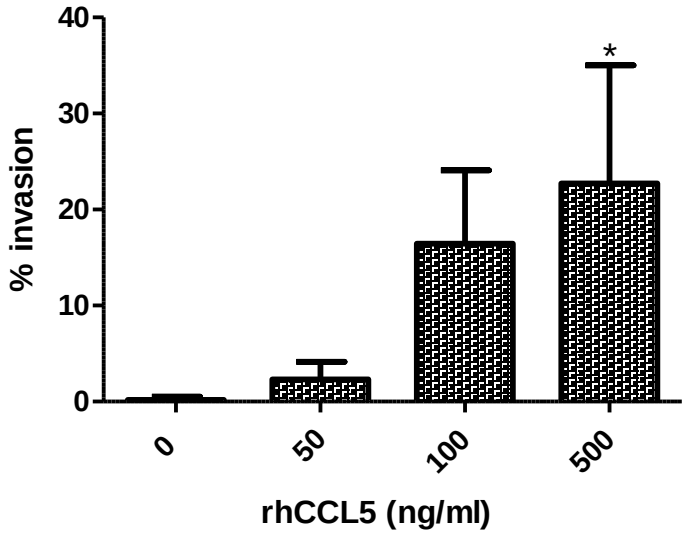


Figure 4.38 Percentage invasion of KYSE30 in 0ng/mL, 50 ng/mL, 100 ng/mL and 500 ng/mL of rhCCL5 after 24hours. Compared with 0 ng/ml rhCCL5, * $p<0.05$.

Chapter 5 Discussion

5.1. Study of downstream regulation of oncogene *GAEC1* and its clinical significance in ESCC

Previous study of *GAEC1* by our group has already confirmed its oncogenic properties(Law, F. *et al.*, 2007). In that study, overexpression of *GAEC1* caused malignant transformation of mouse 3T3 fibroblasts cells. The transforming properties of *GAEC1* are comparable to well known oncogene *H-Ras*, as both *H-Ras* and *GAEC-1* could result in the formation of sarcoma in nude mice at a comparable capacity. Six out of 10 ESCC cell lines were found overexpressing *GAEC1* while 34% of ESCC patient's specimens got *GAEC1* overexpression. Attempt was also made to correlate the *GAEC1* amplification with clinicopathological parameters and prognosis in ESCC. However, no significant correlation was observed in the study(Law, F. *et al.*, 2007). Therefore, current study was conducted to identify the downstream-regulated genes of *GAEC1* and their clinicopathological significance.

ESCC cell line KYSE150, showing stable and consistent *GAEC1* overexpression over non-tumor esophageal cell line NE-1, was used for current study. Suppression of *GAEC1* expression was achieved by RNA interference. RNA interference can be mediated by introduction of chemically synthesized

double-stranded small interfering RNA (siRNA) or transfection of vector containing short hairpin RNA (shRNA) (Rao, D. D. *et al.*, 2009). Suppressing effect by exogenous siRNA is transient and would be diminished after seven to ten rounds of cell division(Tuschl, T., 2002). Compared with exogenous siRNA, vector containing shRNA, as used in the present study, can be continuously synthesized by host cells, making the suppression effect more durable. Transfection of plasmid DNA of vector containing shRNA into the cells allows establishment of cell line with stable target gene suppression. Therefore, vector based RNA interference approach was employed in current study in order to give stable and long-term suppression on expression of *GAEC1* in KYSE150 for consecutive experiments.

From the present study, reduced proliferation rate and increased apoptosis were detected when *GAEC1* expression was suppressed in KYSE150 cells. This indicated that suppressing *GAEC1* expression by RNA interference was an effective approach in reducing the malignant properties of ESCC cells. Similar approach against potential oncogenes has already been applied for the development of gene therapy for various types of cancers (Burnett, J. C. *et al.*, 2011). For example, suppression of *MTA1* in esophageal carcinoma(Qian, H. *et al.*, 2005), alpha-actinin-4 in oral carcinoma(Yamada, S.-i. *et al.*, 2010), osteopontin

in colon carcinoma (Likui, W. *et al.*, 2011) and *EGFR* in hepatocellular carcinoma (Hu, Y. *et al.*, 2011). The application of RNAi approach has been regarded as having great potential for the clinical application of targeted cancer therapy. Many RNAi-based drugs have been developed and demonstrated great promise for anti-cancer therapeutics in preclinical or clinical trials(Wang, Z. *et al.*, 2011). RNAi approach targeting transforming growth factor-β has been employed for development of "cancer vaccine". The vaccine is currently in Phase II clinical assessments against ovarian cancer (clinicaltrials.gov, NCT01309230), melanoma (clinicaltrials.gov, NCT01453361) and colorectal carcinoma (clinicaltrials.gov, NCT01505166). Therefore, this study offered a new approach for developing novel RNAi-based gene therapy targeting oncogene *GAEC1* in the future investigations.

Calpain 10 (*CAPN10*) is one of the members of the mitochondrial calpain system. This system has been shown to promote caspase-independent programmed cell death via mechanism mediated by apoptotic inducing factor (AIF) (Kar, P. *et al.*, 2010). Role of *CAPN10* in insulin-stimulated glucose uptake (Brown, A. E. *et al.*, 2007) and type 2 diabetes (Horikawa, Y., 2006) has been revealed. And expression of *CAPN10* has also been associated with some cancers such as laryngeal cancer(Moreno-Luna, R. *et al.*, 2011), colorectal cancer (Frances, C. P.

et al., 2007) as well as pancreatic cancers(Fong, P.-y. *et al.*, 2010). However, the correlation and functional roles of *CAPN10* in tumorigenesis in ESCC are still not fully understood. In the present study, the RNAi-based suppression of *GAEC1* in KYSE150 resulted in approximately 15-fold of down-regulation of *CAPN10* expression compared with the parental cells. Besides, the 5-year survival rate for ESCC patients with tumors tissue showing low expression of *CAPN10* is significantly lower than those showing high expression of *CAPN10*. This observation is actually similar to the study by Moreno-Luna *et al (Moreno-Luna, R. et al., 2011)* that *CAPN10* genotype 12 was reported to be related with a worse prognosis in laryngeal cancer. Our observation from the low *CAPN10* expression group implied the possibility that the oncogene *GAEC1* overexpression within this group might involve more prominently at the initial stage of molecular carcinogenesis, so that the expression level of *CAPN10* was lower in ESCC at the time of surgical operation. Similar molecular mechanism in carcinogenesis has been demonstrated by fibroblast growth factor-2 in melanoma(Tsunoda, S. *et al.*, 2007) and *KLF4* in cutaneous squamous epithelial neoplasia (Huang, C. C. *et al.*, 2005). To verify this hypothesis, investigation on protein expression of GAEC-1 on ESCC specimen tissues of different stages using GAEC-1-specific antibody (not yet available in the market) is needed in the future. However, it is possible

that the resulting changes in CAPN10 expression level can be due to some other upstream regulators other than GAEC-1 that have not been identified. This can be the later direction of future research for CAPN10. And it is believed that the finding would also pave the path for the further investigation for the roles of *CAPN10* in the molecular pathogenesis of ESCC.

5.2. Study of reversal of tumor transforming properties of *JK-1*

JK-1 was first mapped to chromosome 5p15.1 using a known cancer-related gene CTNND2 as a landmark previously. Chromosome 5p is a region showing strong genomic amplification signal in ESCC cell lines and patient cases by comparative genomic hybridization (CGH) (Cheung, L. *et al.*, 2007; Fatima, S. *et al.*, 2006; Hu, Y. C. *et al.*, 2002; Tang, W. K. *et al.*, 2007; Yen, C. C. *et al.*, 2001). In particular, gain of 5p15 has been associated with adverse stage and undesirable outcome in ESCC patients, including significantly lower survival rate and more distant organ metastasis after surgery. Gain of 5p15 detected by CGH thus has been suggested as one of the prognostic marker for ESCC(Ueno, T. *et al.*, 2002). Apart from *JK-1*, chromosome 5p15 harbors several cancer-related genes, including *CTNND2* (Lu, Q. *et al.*, 1999; Reynolds, A. B. and Roczniak-Ferguson, A., 2004), *JS-1*(Fatima, S. *et al.*, 2006), *hTERT*(Shen, Y. *et al.*, 2008) (5p15.33) and *BASP1*(Moribe, T. *et al.*, 2008) (5p15.1).

Functional studies for *JK-1* have been conducted previously by our group to show its oncogenic properties(Tang, W. K. *et al.*, 2007). Expressing *JK-1* in non-tumor mouse fibroblast NIH 3T3 cells and non-tumor embryonic kidney HEK293 cells using *JK-1* expression vector conferred the non-tumor cells to have higher

cell proliferation rate, ability to form colonies in soft agar as well as foci upon growing to confluence in culture plate. In the present study, in order to examine the potential for *JK-1* to be the therapeutic target at molecular level, RNAi technique for knocking-down gene expression was employed for targeted suppression of *JK-1*.

To study the association of *JK-1* expression with the pathogenesis of ESCC, protein expression of *JK-1* was examined in tissue specimens from ESCC patients by immunohistochemical staining analysis. Previous study of our group has revealed that 30% of ESCC malignant tumors got mRNA overexpression of *JK-1* (Tang, W. K. *et al.*, 2007). In the current study, protein expression of *JK-1* was found elevated in 26.9% of ESCC tumor tissues, which is similar to our previous observation for mRNA expression. Up-regulation of JK-1 occurred in high frequency in non-tumor tissues (93.3%) and pre-malignant tissues (dysplasia and hyperplasia) (87.5%). The high protein expression level of JK-1 in pre-malignant tissues provided evidence for the possible tumor transforming role of *JK-1* at the early stage of pathogenesis in ESCC. JK-1 protein was expressed in high level in non-tumor tissues suggesting the possibility for occurrence of carcinogenesis in these tissues.

In addition, the present study may also offer a chance for the early detection

of the premalignant or precursor lesions for ESCC and thus advices can be given to the patients so that they have earlier awareness and treatment of the disease. Esophageal squamous dysplasia (ESD) is characterized with nuclear atypia, loss of normal cell polarity as well as abnormal tissue maturation without invasion of epithelial cell across the basement membrane(Taylor, P. R. *et al.*, 2013). ESD was first recognized as precancerous feature for ESCC in 1988 (Qiu, S. and Yang, G., 1988). Subsequent study also reported that ESD predicts the development of ESCC(Dawsey, S. M. *et al.*, 1994). Whereas basal cell hyperplasia (BCH) is characterized with increased thickness of basal zone occupying over 15% of the thickness of the epithelium. In Henan Province, one of regions in China with high incidence of ESCC, got over 20% of residents(1574/7381) detected with hyperplasia in a endoscopic study, which implicated hyperplasia as the pre-cancerous lesion for ESCC(He, Z. *et al.*, 2010).

In the present study, high percentage of JK-1 protein expression in pre-malignant lesions was observed. In general, survival rate of ESCC is extremely poor which is largely due to late diagnosis (Tong, D. and Law, S., 2015) and the survival rate can be highly increased if diagnosis is made at earlier stage. For patients with diagnosis made at the early stage T1 ,the 5-year survival rate was found around 87% (Wang, G.-Q. *et al.*, 2004). However, it dropped greatly for

advanced stages - the 5-year survival rates with diagnosis made at the stage of localized , regional and distant tumor were 40.4% , 21.6% and 4.2% respectively (National Cancer Institute, 2011). The present finding of elevated expression of JK-1 in pre-malignant tissues raised the possibility that JK-1 protein expression could be an early biomarker for the detection of risk of incidence and hence reduce the mortality of the disease if earlier treatment is followed. In addition, effective reversal of *in vitro* tumor transforming properties of *JK-1* by the vector-based RNAi method demonstrated in this study might also allow the prevention of development of ESCC in later studies. However, additional study is still needed for understanding molecular mechanisms of *JK-1* in pathogenesis of ESCC.

5.3. Study of reversal of cisplatin-resistance in ESCC cells

Cisplatin is an effective front-line chemotherapeutic drug. It works by causing DNA damage, followed by apoptosis of cancer cells (Basu, A. and Krishnamurthy, S., 2010). Occurrence of resistance to cisplatin is the main challenge in ESCC chemotherapy. Many mechanisms have been suggested contributing to resistance to cisplatin. These mechanisms included blocking in transduction of DNA damaging signal and/or apoptotic signal, development of DNA repair mechanism as well as elimination of cisplatin.

The mechanisms of acquiring cisplatin are multifactorial and may be unique to different types of cancers (Galluzzi, L. *et al.*, 2012; Kartalou, M. and Essigmann, J. M., 2001). In the present study, cisplatin-resistant ESCC cell line SLMT-1/CDDP1R was obtained by repeatedly treating SLMT-1 cells with increasing doses of cisplatin. And the differential expression of genes between parental and cisplatin-resistant cell line was identified using cDNA microarray technology. Similar approach was adopted in some previous studies for identifying the potential molecular targets in ESCC cells with cisplatin-resistance. H. Toshimitsu *et al.* established a cisplatin-resistant subline for ESCC cell line YES-2 and characterized the cisplatin-resistant YES-2 cell line with decreased cisplatin accumulation and frequent under-expression of genes encoding ribosome-related

proteins (Toshimitsu, H. *et al.*, 2004). L. Yu *et al* revealed the increase in autophagy activity in cisplatin-resistant EC109 cells compared with parental EC109 and inhibition on autophagy was able to enhance cytotoxic effect of cisplatin on the resistant cell line(Yu, L. *et al.*, 2014).

In the present study, significant down-regulation of *IGFBP5* was discovered in SLMT-1 cells acquiring cisplatin-resistance by microarray analysis and verified by qPCR. And the causal link of down-regulation of *IGFBP5* and acquisition of cisplatin-resistance was confirmed by using siRNA-based RNA interference. To the best of our knowledge, this is the first study which demonstrated the down-regulation of *IGFBP5* leading to acquisition of cisplatin-resistance in ESCC. Another member of IGFBPs family, *IGFBP3*, showed similar effect in a study conducted by L. Zhao *et al.* Over-expressing *IGFBP3* enhanced sensitivity of KYSE30 cells to cisplatin, and knocking-down of *IGFBP3* by specific siRNA reduced sensitivity of KYSE30 to cisplatin (Zhao, L. *et al.*, 2012).

IGFBPs are known as their inhibitory effect on IGF-stimulated activities by sequestering IGF away from IGF-R (Clemmons, D. R., 1998; Firth, S. M. and Baxter, R. C., 2002). Hyperactivation of IGF signaling pathway has been reported for leading to cisplatin-resistance in ovarian cancer (Eckstein, N. *et al.*, 2009) and lung cancer (Sun, Y. *et al.*, 2012). Improved cisplatin treatment outcome was

observed in inhibition of IGF signaling pathway. Blockade of IGF signaling at IGF-receptor(IGF-1R) was found enhancing cisplatin-induced apoptosis in ESCC cells (Imsumran, A. *et al.*, 2007). The use of monoclonal antibody against IGF-1R accompanying cisplatin improved inhibitory efficacy in small cell lung cancer *in vivo* and *in vitro* in nude mice bearing tumors(Ferté, C. *et al.*, 2013). The mechanism underlying enhancing effect of cisplatin by blockade of IGF signaling pathway was not yet fully understood. However, some previous findings about molecular of action of *IGF-1* signaling pathway by H. Milhavet *et al.* gave out some ideas for it. It was found that DNA repair pathway, *p38 MAP* kinase signaling pathway, is mediated by *IGF-1* in fibroblast cells. As anti-tumor effect of cisplatin relies on its DNA damage properties, DNA repairing mediated by *IGF-1* might lead to reduced sensitivity to cisplatin(Héron-Milhavet, L. *et al.*, 2001). In addition, *IGF-1* rescued cells from apoptosis by inducing *p53* protein degradation upon DNA damage (Héron-Milhavet, L. and LeRoith, D., 2002). *p53* is a key regulator in DNA damaging signaling. Degradation of p53 protein might aid the damaged cells escaped from apoptosis and thus cisplatin-resistance.

In order to assess whether downregulation of *IGFBP5* results in hyperactiviation of IGF-signaling pathway and in turn confers cisplatin-resistance to ESCC, the investigation on status of IGF-signaling in SLMT-1/CDDP1R

compared with parental SLMT-1 is suggested in future studies.

Suppression on *IGFBP5* by specific siRNA resulted in decrease in sensitivity to cisplatin treatment in the present study, this indicated the potential of reversal of cisplatin-resistance by restoring *IGFBP5* level in SLMT-1/CDDP1R cells. *IGFBP5* expression vector was thus transfected into SLMT-1/CDDP1R cells and was evaluated for the efficacy of reversing the resistance. Significant reduction in cell viability was shown in cytotoxicity assay of CDDP in cisplatin-resistant ESCC cells after transfection of *IGFBP5* expressing vector. This offered the prospect of overcoming cisplatin-resistance in cisplatin-treatment if the approach can be extended to the animal or human studies in future.

5.4. Study of anti-cancer effect of 91b1 on ESCC cells

In the current study, cytotoxic effect of 91b1 on five ESCC cell lines (KYSE150, KYSE450, KYSE510, HKESC-4, and KYSE30) was evaluated by MTS cytotoxicity assay. 91b1 exerted cytotoxic effect through inhibiting MTS activity by 50% in the five ESCC cells that are comparable to cisplatin. In addition, 91b1 demonstrated less cytotoxicity to non-tumor cell line NE-3 and HEK293 than cisplatin by 1.6 and 2.1 times respectively. Hence, the current results provide the first evidence about the anti-cancer potential of 91b1 and thus it was further investigated about its anti-cancer mechanisms in the later part of this study.

Among the five ESCC cell lines, KYSE150, KYSE450 and HKESC-4 were found to show more anti-cancer effect with the treatment of 91b1 than cisplatin. In particular, KYSE150 which was the least sensitive to cisplatin treatment (MTS_{50}:13.16μg/mL) showed better cytotoxicity with the treatment of 91b1 (MTS_{50}: 4.55μg/mL). KYSE150 cells treated with 91b1 were then examined by cDNA microarray analysis for the investigation of molecular actions of 91b1. A C-C chemokine, *CCL5* was found greatly downregulated in 91b1-treated KYSE150 cells with the highest fold change. *CCL5*, also named as *RANTES*, regulated upon activation, normal T cell and secreted, is one of the members in CC-chemokine family (Martin, L. *et al.*, 2001). It was found mediating its

biological effect by activating G protein-coupled receptors CCR1, CCR3 and CCR5 with CCR5 as the dominant receptor (Vaday, G. G. *et al.*, 2006; Wang, S.-W. *et al.*, 2012). The most important role for the interaction CCL5 and its receptor CCR5 in tumor development is the regulation of metastasis process. Mechanism of metastasis mediated by elevated level of CCL5 has not yet been fully understood. However, several reports have demonstrated the influence of the CCL5/CCR5 activity on invasion. Secretion of CCL5 by stromal cells in bone marrow was found enhancing the invasion ability of hepatocellular cancinoma cells(Bai, H. *et al.*, 2014). And CCL5 also promoted migration and invasion of lung cancer cells (Huang, C.-Y. *et al.*, 2009) and oral cancer cells (Chuang, J. Y. *et al.*, 2009)

In present study, trans-well matrigel invasion assay was performed to examine the potential functions of CCL5 in ESCC. CCL5 (50 -500 ng/mL) induced invasion ability of KYSE30 in the matrigel trans-well invasion assay, implying that CCL5 can enhance the invasion ability of ESCC cancer cells. From the present study, CCL5 expression in turn can be suppressed by 91b1, thus the overall findings illustrated the potential of 91b1 in suppressing the invasion of ESCC cells through CCL5 suppression.

In the current study, 87.5% of ESCC cell lines expressed higher level of

CCL5 than the non-tumor esophageal epithelium cells. The elevated expression of CCL5 was also observed in ESCC patient tumor specimens by IHC – 76.9% of ESCC tumor tissues showed high expression level for CCL5. Notably, the pre-malignant tissues also showed high frequency (62.5%) with high expression level of CCL5. This implied that CCL5 may be involved in the early stage of carcinogenesis of ESCC , playing role in transformation of pre-malignant lesions to tumor as reported in oral squamous cell carcinoma (Chen, D. *et al.*, 2011). Some previous studies suggested that different organs or tissues may secrete different chemokines (including CCL5) and express lectins and integrins of specific types which favor the binding of cancer cells to the organs from the circulation and result in metastasis (Cooper, C. R. *et al.*, 2000; Karmakar, S. and Mukherjee, R., 2003; Langley, R. R. and Fidler, I. J., 2011). This suggests that the overexpression of CCL5 in other tissues may also increase the risk of metastasis to other organs or tissues. Thus according to the results of the present study that 91b1 can suppress the expression of CCL5, the anti-cancer actions of 91b1 may also involve the suppression of metastasis of cancer cells to other tissue sites.

Chapter 6 Summary of Conclusions

Study of downstream regulation of oncogene *GAEC1* and its clinical significance on ESCC

Suppression of *GAEC1* expression by RNAi approach using pSilencer-P3-4 resulted in reduced ESCC cell proliferation rate and increased apoptotic population in ESCC cells. In this study, *in vitro* suppression on *GAEC1* expression resulted in down-regulation of CAPN10 expression. The low expression of CAPN10 predicted the poor survival rate in ESCC patients.

Study of reversal of tumor transforming properties of *JK-1*

Suppression of *JK-1* expression by RNAi approach resulted in decreased ESCC cell proliferation rate, reduced cell migration, reversal of tumor transforming ability including reduced anchorage-independent growth ability and gain of contact inhibition. Protein expression level of JK-1 was found to be more frequent in tissues at pre-malignant stage compared with tumor tissues, implying that *JK-1* plays a more important role during the initial stage of tumor formation.

Study of reversal of cisplatin-resistance in ESCC cells

This is the first study to showed down-regulation of *IGFBP5* in ESCC cells with cisplatin-resistance. Knockdown of *IGFBP5* in parental SLMT-1 cells confirmed that *IGFBP5* suppression is one of the mechanisms for ESCC cells acquiring cisplatin-resistance. And the cisplatin-resistance phenotype can be reversed by upregulation of *IGFBP5*.

Study of anti-cancer effect of 91b1 on ESCC cells

The novel quinoline compound 91b1 demonstrated strong cytotoxic effect to ESCC cells with relatively lower cytotoxicity to non-tumor cells compared to CDDP. 91b1 suppressed CCL5 mRNA and protein expression. CCL5 was found frequently upregulated in ESCC cell lines and tumor tissues. It also enhanced invasive ability of ESCC cells *in vitro*.

The overall results of above suggest the novel molecular and pharmacological approaches for the possible treatment of ESCC at the experimental stages. The long-term development of the above treatment methods in animal tests before clinical trials is absolutely required in the long run. The future findings of the related methods hopefully can be extended to other cancer types in future.

APPENDICES

Appendix A - Information of *GAEC1*

(I) General Information for *GAEC1*

Gene ID:	**100126794**
Gene Symbol	*GAEC1*
Definition	gene amplified in esophageal cancer 1
Source	Homo sapiens (human)
Definition	Homo sapiens hypothetical protein FLJ20152, transcript variant 2, mRNA
Location	Chromosome 7q22.1
Entire length of gene	2052bp
Coding sequence (CDS)	519-848
Predicted protein	109 amino acids

(II) Coding sequence of *GAEC1*

"ATGGCCTGGGCCTGGGCTCGTGACCTTCCCTGGAGGCCATGGTGGTGGTGGTG
GTGGTGGCAGGTGTGTTCCCGTCTCCACCCCTCAGGGAAGAAGCAAGTTCCAGG
AGCCTGGACTTTTGCCCTGCGCACTCTCTGTGTCAGATGGGGAGACAGCCATGA
GGACAGACACTTGAAGTACCCTATAACTGGCACCATGCAAGAGAGACAGGCAGG
AGGAGACTGTGGGAGCTCAGAGGAGACACTTGTGCTGGCCTGGATCAGGGAGAA
AGGAGGGGTGTCGGGAGGCTTCCTGGAAGAAGTGGCTTCTGGATTAATTATTGA
AGTGTGA"

(III) Protein sequence after *GAEC1* translation

MAWAWARDLPWRPWWWWWWWQVCSRLHPSGKKQVPGAWTFALRTLCVRWGDSHE
DRHLKYPITGTMQERQAGGDCGSSEETLVLAWIREKGGVSGGFLEEVASGIIIE
V

Appendix B - Information of *JK-1*

(I) General Information for *JK-1*

Accession no.:	NM_019000
Gene Symbol	*JK-1* (FAM143B)
Definition	Homo sapiens family with sequence similarity 134, member B (FAM134B), transcript variant 2, mRNA.
Source	Homo sapiens (human)
Definition	Homo sapiens hypothetical protein FLJ20152, transcript variant 2, mRNA
Location	Chromosome 5p15.1
Entire length of gene	3184bp
Coding sequence (CDS)	387-1457
Predicted protein	356 amino acids

(II) Coding sequence of *JK-1*

"ATGCCTGAAGGTGAAGACTTTGGACCAGGCAAAAGCTGGGAAGTTATCAATTC
CAAACCAGATGAAAGACCCAGGCTCAGCCACTGTATTGCAGAATCATGGATGAA
TTTCAGCATATTTCTTCAAGAAATGTCTCTTTTTAAACAGCAGAGCCCTGGCAA
GTTTTGTCTCCTGGTCTGTAGTGTGTGCACATTTTTTACGATCTTGGGAAGTTA
CATTCCTGGGGTTATACTCAGCTATCTACTGTTACTGTGTGCATTTTTGTGTCC
ATTGTTTAAATGTAATGATATTGGACAAAAAATTTACAGCAAAATTAAGTCAGT
TCTGCTGAAACTGGATTTTGGAATTGGAGAATATATTAATCAGAAGAAACGTGA
GAGATCTGAAGCAGACAAAGAAAAAAGTCACAAAGATGACAGTGAATTAGACTT
TTCAGCTCTTTGTCCTAAGATTAGCCTCACGGTTGCTGCCAAAGAGTTATCTGT
GTCTGACACAGACGTCTCAGAGGTATCCTGGACTGATAATGGGACCTTCAACCT
TTCAGAAGGATACACTCCACAGACAGACACTTCTGATGATCTTGACCGACCCAG
TGAGGAAGTTTTCTCTAGAGATCTTTCAGATTTTCCATCTCTAGAAAATGGCAT
GGGAACAAATGATGAAGATGAATTAAGCCTTGGTTTGCCCACTGAGCTCAAGAG
AAAGAAGGAACAGTTGGACAGTGGTCACAGACCAAGCAAAGAGACGCAATCAGC
AGCTGGTCTCACCCTTCCTCTGAACAGTGACCAAACCTTTCACCTGATGAGCAA
CCTGGCTGGGGATGTTATCACAGCTGCAGTGACTGCAGCTATCAAAGACCAGTT
AGAGGGTGTGCAGCAAGCACTTTCTCAGGCTGCCCCCATCCCAGAAGAGGACAC
AGACACTGAAGAAGGTGATGACTTTGAACTACTTGACCAGTCAGAGCTGGATCA

AATTGAGAGTGAATTGGGACTTACACAAGACCAGGAAGCAGAAGCACAGCAAAA
TAAGAAGTCTTCAGGTTTCCTTTCAAATCTGCTGGGAGGCCATTAA”

(III) Protein sequence after *JK-1* translation

“MPEGEDFGPGKSWEVINSKPDERPRLSHCIAESWMNFSIFLQEMSLFKQQSPG
KFCLLVCSVCTFFTILGSYIPGVILSYLLLLCAFLCPLFKCNDIGQKIYSKIKS
VLLKLDFGIGEYINQKKRERSEADKEKSHKDDSELDFSALCPKISLTVAAKELS
VSDTDVSEVSWTDNGTFNLSEGYTPQTDTSDDLDRPSEEVFSRDLSDFPSLENG
MGTNDEDELSLGLPTELKRKKEQLDSGHRPSKETQSAAGLTLPLNSDQTFHLMS
NLAGDVITAAVTAAIKDQLEGVQQALSQAAPIPEEDTDTEEGDDFELLDQSELD
QIESELGLTQDQEAEAQQNKKSSGFLSNLLGGH"

Appendix C - Information of IGFBP5/pcMV3-C-Myc

(I) Information of *IGFBP5* cDNA clone

Gene Bank Ref. ID:	NM_000599.3
cDNA Size:	864
cDNA Description:	ORF Clone of Homo sapiens insulin-like growth factor binding protein 5 DNA
Gene Synonym:	*IGFBP5*
Species:	Human
Vector:	pCMV3-C-Myc
Vector size	6888
Restriction Site:	KpnI + XbaI
Tag Sequence:	Myc Tag Sequence: GAGCAGAAACTCATCTCAGAAGAGGATCTG

(II) Sequences of IGFBP-5/pcMV3-C-Myc, *IGFBP5* expression vector

```
1       GACGGATCGG GAGATCTCCC GATCCCCTAT GGTGCACTCT CAGTACAATC TGCTCTGATG
61      CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
121     CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
181     TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG AGTACATTTA TATTGGCTCA TGTCCAATAT
241     GACCGCCATG TTGACATTGA TTATTGACTA GTTATTAATA GTAATCAATT ACGGGGTCAT
301     TAGTTCATAG CCCATATATG GAGTTCCGCG TTACATAACT TACGGTAAAT GGCCCGCCTG
361     GCTGACCGCC CAACGACCCC CGCCCATTGA CGTCAATAAT GACGTATGTT CCCATAGTAA
421     CGCCAATAGG GACTTTCCAT TGACGTCAAT GGGTGGAGTA TTTACGGTAA ACTGCCCACT
481     TGGCAGTACA TCAAGTGTAT CATATGCCAA GTCCGCCCCC TATTGACGTC AATGACGGTA
541     AATGGCCCGC CTGGCATTAT GCCCAGTACA TGACCTTACG GGACTTTCCT ACTTGGCAGT
601     ACATCTACGT ATTAGTCATC GCTATTACCA TGGTGATGCG GTTTTGGCAG TACACCAATG
661     GGCGTGGATA GCGGTTTGAC TCACGGGGAT TTCCAAGTCT CCACCCCATT GACGTCAATG
721     GGAGTTTGTT TTGGCACCAA AATCAACGGG ACTTTCCAAA ATGTCGTAAT AACCCCGCCC
781     CGTTGACGCA AATGGGCGGT AGGCGTGTAC GGTGGGAGGT CTATATAAGC AGAGCTCGTT
841     TAGTGAACCG TCAGATCCTC ACTCTCTTCC GCATCGCTGT CTGCGAGGGC CAGCTGTTGG
901     GCTCGCGGTT GAGGACAAAC TCTTCGCGGT CTTTCCAGTA CTCTTGGATC GGAAACCCGT
961     CGGCCTCCGA ACGGTACTCC GCCACCGAGG GACCTGAGCG AGTCCGCATC GACCGGATCG
1021    GAAAACCTCT CGAGAAAGGC GTCTAACCAG TCACAGTCGC AAGGTAGGCT GAGCACCGTG
1081    GCGGGCGGCA GCGGGTGGCG GTCGGGGTTG TTTCTGGCGG AGGTGCTGCT GATGATGTAA
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1141 TTAAAGTAGG CGGTCTTGAG ACGGCGGATG GTCGAGGTGA GGTGTGGGTT TAGTGAACCG
1201 TCAGATCCTC ACTCTCTTCC GCATCGCTGT CTGCGAGGGC CAGCTGTCAG GCTTGAGATC
1261 CAGCTGTTGG GGTGAGTACT CCCTCTCAAA AGCGGGCATT ACTTCTGCGC TAAGATTGTC
1321 AGTTTCCAAA AACGAGGAGG ATTTGATATT CACCTGGCCC GATCTGGCCA TACACTTGAG
1381 TGACAATGAC ATCCACTTTG CCTTTCTCTC CACAGGTGTC CACTCCCAGG TCCAAGTTTA
1441 AACTTTAATA CGACTCACTA TAGGGGCCGC CACCAAGCTT GGTACATGGT GTTGCTCACC
1501 GCGGTCCTCC TGCTGCTGGC CGCCTATGCG GGGCCGGCCC AGAGCCTGGG CTCCTTCGTG
1561 CACTGCGAGC CCTGCGACGA GAAAGCCCTC TCCATGTGCC CCCCCAGCCC CCTGGGCTGC
1621 GAGCTGGTCA AGGAGCCGGG CTGCGGCTGC TGCATGACCT GCGCCCTGGC CGAGGGGCAG
1681 TCGTGCGGCG TCTACACCGA GCGCTGCGCC AGGGGCTGCG CTGCCTCCCC CGGCAGGACG
1741 AGGAGAAGCC GCTGCACGCC CTGCTGCACG GCCGCGGGGT TTGCCTCAAC GAAAAGAGCT
1801 ACCGCGAGCA AGTCAAGATC GAGAGAGACT CCCGTGAGCA CGAGGAGCCC ACCACCTCTG
1861 AGATGGCCGA GGAGACCTAC TCCCCCAAGA TCTTCCGGCC CAAACACACC CGCATCTCCG
1921 AGCTGAAGGC TGAAGCAGTG AAGAAGGACC GCAGAAAGAA GCTGACCCAG TCCAAGTTTG
1981 TCGGGGGAGC CGAGAACACT GCCCACCCCC GGATCATCTC TGCACCTGAG ATGAGACAGG
2041 AGTCTGAGCA GGGCCCCTGC CGCAGACACA TGGAGGCTTC CCTGCAGGAG CTCAAAGCCA
2101 GCCCACGCAT GGTGCCCCGT GCTGTGTACC TGCCCAATTG TGACCGCAAA GGATTCTACA
2161 AGAGAAAGCA GTGCAAACCT TCCCGTGGCC GCAAGCGTGG CATCTGCTGG TGCGTGGACA
2221 AGTACGGGAT GAAGCTGCCA GGCATGGAGT ACGTTGACGG GGACTTTCAG TGCCACACCT
2281 TCGACAGCAG CAACGTTGAG GGGGGTGGAG GCTCTGAGCA GAAACTCATC TCAGAAGAGG
2341 ATCTGTAAAC TCGAGTCTAG AGCGGCCGCC GAATTCGGGC CCGTTTAAAC CCGCTGATCA
2401 GCCTCGACTG TGCCTTCTAG TTGCCAGCCA TCTGTTGTTT GCCCCTCCCC CGTGCCTTCC
2461 TTGACCCTGG AAGGTGCCAC TCCCACTGTC CTTTCCTAAT AAAATGAGGA AATTGCATCG
2521 CATTGTCTGA GTAGGTGTCA TTCTATTCTG GGGGGTGGGG TGGGGCAGGA CAGCAAGGGG
2581 GGGCAGGACA GCAAGGGGGA GGATTGGGAA GACAATAGCA GGCATGCTGG GGATGCGGTG
2641 GGCTCTATGG CTTCTGAGGC GGAAAGAACC AGCTGGGGCT CTAGGGGGTA TCCCCACGCG
2701 CCCTGTAGCG GCGCATTAAG CGCGGCGGGT GTGGTGGTTA CGCGCAGCGT GACCGCTACA
2761 CTTGCCAGCG CCCTAGCGCC CGCTCCTTTC GCTTTCTTCC CTTCCTTTCT CGCCACGTTC
2821 GCAGGCTTTC CCCGTCAAGC TCTAAATCGG GGGCTCCCTT TAGGGTTCCG ATTTAGTGCT
2881 TTACGGCACC TCGACCCCAA AAAACTTGAT TAGGGTGATG GTTCACGTAG TGGGCCATCG
2941 CCCTGATAGA CGGTTTTTCG CCCTTTGACG TTGGAGTCCA CGTTCTTTAA TAGTGGACTC
3001 TTGTTCCAAA CTGGAACAAC ACTCAACCCT ATCTCGGTCT ATTCTTTTGA TTTATAAGGG
3061 ATTTTGCCGA TTTCGGCCTA TTGGTTAAAA AATGAGCTGA TTTAACAAAA ATTTAACGCG
3121 AATTAATTCT GTGGAATGTG TGTCAGTTAG GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG
3181 GCAGAAGTAT GCAAAGCATG CATCTCAATT AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG
3241 GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCATAGTCC
3301 CGCCCCTAAC TCCGCCCATC CCGCCCCTAA CTCCGCCCAG TTCCGCCCAT TCTCCGCCCC
3361 ATGGCTGACT AATTTTTTTT ATTTATGCAG AGGCCGAGGC CGCCTCTGCC TCTGAGCTAT

3421 TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG GCCTAGGCTT TTGCAAAAAG CTCTCGGGAG

3481 CTTGTATATC CATTTTCGGA TCTGATCAGC ACGTGATGAA AAAGCCTGAA CTCACCGCGA

3541 CGTCTGTCGA GAAGTTTCTG ATCGAAAAGT TCGACAGCGT CTCCGACCTG ATGCAGCTCT

3601 CGGAGGGCGA AGAATCTCGT GCTTTCAGCT TCGATGTAGG AGGGCGTGGA TATGTCCTGC

3661 GGGTAAATAG CTGCGCCGAT GGTTTCTACA AAGATCGTTA TGTTTATCGG CACTTTGCAT

3721 CGGCCGCGCT CCCGATTCCG GAAGTGCTTG ACATTGGGGA ATTCAGCGAG AGCCTGACCT

3781 ATTGCATCTC CCGCCGTGCA CAGGGTGTCA CGTTGCAAGA CCTGCCTGAA ACCGAACTGC

3841 CCGCTGTTCT GCAGCCGGTC GCGGAGGCCA TGGATGCGAT CGCTGCGGCC GATCTTAGCC

3901 AGACGAGCGG GTTCGGCCCA TTCGGACCGC AAGGAATCGG TCAATACACT ACATGGCGTG

3961 ATTTCATATG CGCGATTGCT GATCCCCATG TGTATCACTG GCAAACTGTG ATGGACGACA

4021 CCGTCAGTGC GTCCGTCGCG CAGGCTCTCG ATGAGCTGAT GCTTTGGGCC GAGGACTGCC

4081 CCGAAGTCCG GCACCTCGTG CACGCGGATT TCGGCTCCAA CAATGTCCTG ACGGACAATG

4141 GCCGCATAAC AGCGGTCATT GACTGGAGCG AGGCGATGTT CGGGGATTCC CAATACGAGG

4201 TCGCCAACAT CTTCTTCTGG AGGCCGTGGT TGGCTTGTAT GGAGCAGCAG ACGCGCTACT

4261 TCGAGCGGAG GCATCCGGAG CTTGCAGGAT CGCCGCGGCT CCGGGCGTAT ATGCTCCGCA

4321 TTGGTCTTGA CCAACTCTAT CAGAGCTTGG TTGACGGCAA TTTCGATGAT GCAGCTTGGG

4381 CGCAGGGTCG ATGCGACGCA ATCGTCCGAT CCGGAGCCGG GACTGTCGGG CGTACACAAA

4441 TCGCCCGCAG AAGCGCGGCC GTCTGGACCG ATGGCTGTGT AGAAGTACTC GCCGATAGTG

4501 GAAACCGACG CCCCAGCACT CGTCCGAGGG CAAAGGAATA GCACGTGCTA CGAGATTTCG

4561 ATTCCACCGC CGCCTTCTAT GAAAGGTTGG GCTTCGGAAT CGTTTTCCGG GACGCTGGCT

4621 GGATGATCCT CCAGCGCGGG GATCTCATGC TGGAGTTCTT CGCCCACCCC AACTTGTTTA

4681 TTGCAGCTTA TAATGGTTAC AAATAAAGCA ATAGCATCAC AAATTTCACA AATAAAGCAT

4741 TTTTTTCACT GCATTCTAGT TGTGGTTTGT CCAAACTCAT CAATGTATCT TATCATGTCT

4801 GTATACCGTC GACCTCTAGC TAGAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT

4861 GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA AAGTGTAAAG

4921 CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC GTTGCGCTCA CTGCCCGCTT

4981 TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT CGGCCAACGC GCGGGGAGAG

5041 GCGGTTTGCG TATTGGGCGC TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTCG

5101 TTCGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT

5161 CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA

5221 AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC CCCTGACGAG CATCACAAAA

5281 ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC

5341 CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT

5401 CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA

5461 GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG

5521 ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT

5581 CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA GGCGGTGCTA

5641 CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGAACAGTA TTTGGTATCT

5701 GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC

5761 AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA

5821 AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGAGCGCGGA ACCCCTATTT

5881 GTTTATTTTT CTAAATACAT TCAAATATGT ATCCGCTCAT GAATTAATTC TTAGAAAAAC

5941 TCATCGAGCA TCAAATGAAA CTGCAATTTA TTCATATCAG GATTATCAAT ACCATATTTT

6001 TGAAAAAGCC GTTTCTGTAA TGAAGGAGAA AACTCACCGA GGCAGTTCCA TAGGATGGCA

6061 AGATCCTGGT ATCGGTCTGC GATTCCGACT CGTCCAACAT CAATACAACC TATTAATTTC

6121 CCCTCGTCAA AAATAAGGTT ATCAAGTGAG AAATCACCAT GAGTGACGAC TGAATCCGGT

6181 GAGAATGGCA AAAGTTTATG CATTTCTTTC CAGACTTGTT CAACAGGCCA GCCATTACGC

6241 TCGTCATCAA AATCACTCGC ATCAACCAAA CCGTTATTCA TTCGTGATTG CGCCTGAGCG

6301 AGACGAAATA CGCGATCGCT GTTAAAAGGA CAATTACAAA CAGGAATCGA ATGCAACCGG

6361 CGCAGGAACA CTGCCAGCGC ATCAACAATA TTTTCACCTG AATCAGGATA TTCTTCTAAT

6421 ACCTGGAATG CTGTTTTCCC AGGGATCGCA GTGGTGAGTA ACCATGCATC ATCAGGAGTA

6481 CGGATAAAAT GCTTGATGGT CGGAAGAGGC ATAAATTCCG TCAGCCAGTT TAGTCTGACC

6541 ATCTCATCTG TAACATCATT GGCAACGCTA CCTTTGCCAT GTTTCAGAAA CAACTCTGGC

6601 GCATCGGGCT TCCCATACAA TCGATAGATT GTCGCACCTG ATTGCCCGAC ATTATCGCGA

6661 GCCCATTTAT ACCCATATAA ATCAGCATCC ATGTTGGAAT TTAATCGCGG CCTAGAGCAA

6721 GACGTTTCCC GTTGAATATG GCTCATAACA CCCCTTGTAT TACTGTTTAT GTAAGCAGAC

6781 AGTTTTATTG TTCATGACCA AAATCCCTTA ACGTGAGTTT TCGTTCCACT GAGCGTCAGA

6841 CCCCGTAGAA ATCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTC

Appendix D - Information of pcMv/hygro-negative control vector

(I) Information of pcMv/hygro-negative control vector

Vector Name	**pCMV / hygro-Myc**
Vector Size	5558bp
Vector Type	Mammalian Expression Vector
Expression Method	Constitutive, Stable / Transient
Promoter	CMV
Antibiotic Resistance	Ampicillin
Selection In Mammalian Cells	Hygromycin
Protein Tag	GAGCAGAAACTCATCTCAGAAGAGGATCTG
Sequencing Primer	Forward:T7(TAATACGACTCACTATAGGG) Reverse:BGH(TAGAAGGCACAGTCGAGG)

(IV) Sequences of pcMv/hygro-negative control vector

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1       GACGGATCGG GAGATCTCCC GATCCCCTAT GGTGCACTCT CAGTACAATC TGCTCTGATG
61      CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
121     CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
181     TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
241     GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
301     TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
361     CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
421     ATTGACGTCA ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
481     ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
541     ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
601     TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG
661     ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
721     AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
781     GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
841     CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGG
901     CCGCCACCAA GCTTGGTACC GCTGAGCAGA AACTCATCTC AGAAGAGGAT CTGTAAAGGG
961     CCCGTTTAAA CCCGCTGATC AGCCTCGACT GTGCCTTCTA GTTGCCAGCC ATCTGTTGTT
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1021 TGCCCCTCCC CCGTGCCTTC CTTGACCCTG GAAGGTGCCA CTCCCACTGT CCTTTCCTAA

1081 TAAAATGAGG AAATTGCATC GCATTGTCTG AGTAGGTGTC ATTCTATTCT GGGGGGTGGG

1141 GTGGGGCAGG ACAGCAAGGG GGAGGATTGG GAAGACAATA GCAGGCATGC TGGGGATGCG

1201 GTGGGCTCTA TGGCTTCTGA GGCGGAAAGA ACCAGCTGGG GCTCTAGGGG GTATCCCCAC

1261 GCGCCCTGTA GCGGCGCATT AAGCGCGGCG GGTGTGGTGG TTACGCGCAG CGTGACCGCT

1321 ACACTTGCCA GCGCCCTAGC GCCCGCTCCT TTCGCTTTCT TCCCTTCCTT TCTCGCCACG

1381 TTCGCCGGCT TTCCCCGTCA AGCTCTAAAT CGGGGGCTCC CTTTAGGGTT CCGATTTAGT

1441 GCTTTACGGC ACCTCGACCC CAAAAAACTT GATTAGGGTG ATGGTTCACG TAGTGGGCCA

1501 TCGCCCTGAT AGACGGTTTT TCGCCCTTTG ACGTTGGAGT CCACGTTCTT TAATAGTGGA

1561 CTCTTGTTCC AAACTGGAAC AACACTCAAC CCTATCTCGG TCTATTCTTT TGATTTATAA

1621 GGGATTTTGC CGATTTCGGC CTATTGGTTA AAAAATGAGC TGATTTAACA AAAATTTAAC

1681 GCGAATTAAT TCTGTGGAAT GTGTGTCAGT TAGGGTGTGG AAAGTCCCCA GGCTCCCCAG

1741 CAGGCAGAAG TATGCAAAGC ATGCATCTCA ATTAGTCAGC AACCAGGTGT GGAAAGTCCC

1801 CAGGCTCCCC AGCAGGCAGA AGTATGCAAA GCATGCATCT CAATTAGTCA GCAACCATAG

1861 TCCCGCCCCT AACTCCGCCC ATCCCGCCCC TAACTCCGCC CAGTTCCGCC CATTCTCCGC

1921 CCCATGGCTG ACTAATTTTT TTTATTTATG CAGAGGCCGA GGCCGCCTCT GCCTCTGAGC

1981 TATTCCAGAA GTAGTGAGGA GGCTTTTTTG GAGGCCTAGG CTTTTGCAAA AAGCTCCCGG

2041 GAGCTTGTAT ATCCATTTTC GGATCTGATC AGCACGTGAT GAAAAAGCCT GAACTCACCG

2101 CGACGTCTGT CGAGAAGTTT CTGATCGAAA AGTTCGACAG CGTCTCCGAC CTGATGCAGC

2161 TCTCGGAGGG CGAAGAATCT CGTGCTTTCA GCTTCGATGT AGGAGGGCGT GGATATGTCC

2221 TGCGGGTAAA TAGCTGCGCC GATGGTTTCT ACAAAGATCG TTATGTTTAT CGGCACTTTG

2281 CATCGGCCGC GCTCCCGATT CCGGAAGTGC TTGACATTGG GGAATTCAGC GAGAGCCTGA

2341 CCTATTGCAT CTCCCGCCGT GCACAGGGTG TCACGTTGCA AGACCTGCCT GAAACCGAAC

2401 TGCCCGCTGT TCTGCAGCCG GTCGCGGAGG CCATGGATGC GATCGCTGCG GCCGATCTTA

2461 GCCAGACGAG CGGGTTCGGC CCATTCGGAC CGCAAGGAAT CGGTCAATAC ACTACATGGC

2521 GTGATTTCAT ATGCGCGATT GCTGATCCCC ATGTGTATCA CTGGCAAACT GTGATGGACG

2581 ACACCGTCAG TGCGTCCGTC GCGCAGGCTC TCGATGAGCT GATGCTTTGG GCCGAGGACT

2641 GCCCCGAAGT CCGGCACCTC GTGCACGCGG ATTTCGGCTC CAACAATGTC CTGACGGACA

2701 ATGGCCGCAT AACAGCGGTC ATTGACTGGA GCGAGGCGAT GTTCGGGGAT TCCCAATACG

2761 AGGTCGCCAA CATCTTCTTC TGGAGGCCGT GGTTGGCTTG TATGGAGCAG CAGACGCGCT

2821 ACTTCGAGCG GAGGCATCCG GAGCTTGCAG GATCGCCGCG GCTCCGGGCG TATATGCTCC

2881 GCATTGGTCT TGACCAACTC TATCAGAGCT TGGTTGACGG CAATTTCGAT GATGCAGCTT

2941 GGGCGCAGGG TCGATGCGAC GCAATCGTCC GATCCGGAGC CGGGACTGTC GGGCGTACAC

3001 AAATCGCCCG CAGAAGCGCG GCCGTCTGGA CCGATGGCTG TGTAGAAGTA CTCGCCGATA

3061 GTGGAAACCG ACGCCCCAGC ACTCGTCCGA GGGCAAAGGA ATAGCACGTG CTACGAGATT

3121 TCGATTCCAC CGCCGCCTTC TATGAAAGGT TGGGCTTCGG AATCGTTTTC CGGGACGCCG

3181 GCTGGATGAT CCTCCAGCGC GGGGATCTCA TGCTGGAGTT CTTCGCCCAC CCCAACTTGT

3241 TTATTGCAGC TTATAATGGT TACAAATAAA GCAATAGCAT CACAAATTTC ACAAATAAAG

3301 CATTTTTTTC ACTGCATTCT AGTTGTGGTT TGTCCAAACT CATCAATGTA TCTTATCATG

3361 TCTGTATACC GTCGACCTCT AGCTAGAGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG

3421 TGTGAAATTG TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA

3481 AAGCCTGGGG TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG

3541 CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA

3601 GAGGCGGTTT GCGTATTGGG CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG

3661 TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG

3721 AATCAGGGGA TAACGCAGGA AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC

3781 GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA

3841 AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT

3901 TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC

3961 TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG CGCTTTCTCA TAGCTCACGC TGTAGGTATC

4021 TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAGC

4081 CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT

4141 TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG

4201 CTACAGAGTT CTTGAAGTGG TGGCCTAACT ACGGCTACAC TAGAAGAACA GTATTTGGTA

4261 TCTGCGCTCT GCTGAAGCCA GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA

4321 AACAAACCAC CGCTGGTAGC GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA

4381 AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA

4441 ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT

4501 TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA

4561 GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA

4621 TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA CCATCTGGCC

4681 CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA

4741 ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC

4801 AGTCTATTAA TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA

4861 ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT

4921 TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG

4981 CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTTATCAC

5041 TCATGGTTAT GGCAGCACTG CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT

5101 CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCGG CGACCGAGTT

5161 GCTCTTGCCC GGCGTCAATA CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC

5221 TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT

5281 CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA

5341 GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA

5401 CACGGAAATG TTGAATACTC ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG

5461 GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG

5521 TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTC

Appendix E - Reagents and solutions

(I) Preparation of LB broth

About 2 g of LB powder (Sigma,USA) was added to 100 mL of double-distilled water and autoclaved for about 20 mins(Gibco,USA).

(II) Preparation of phosphate buffered saline (PBS) buffer

About 8 g of sodium chloride (NaCl, Sigma,USA), 0.2 g of potassium chloride (KCl, Sigma), 1.44 g of disodium hydrogen phosophate (Na_2HPO_4, Sigm, USA) and 0.24 g of potassium dihydrogen phosphate (KH_2PO_4, Sigma, USA) were added with 1 L of double-distilled water to give 1x of PBS buffer with pH ranged from 7.2 to 7.4. The PBS buffer was autoclaved for about 20 mins before use.

(III) Preparation of geneticin, G-418 sulfate

About 5 g of geneticin, G-418 sulfate (Gibco,USA) was dissolved in 100 mL of distilled water to give a final working concentration of 50 mg/mL.

(IV) Preparation of 10x *tris*-borate-EDTA (TBE) buffer

TBE buffer was used for the agarose gel electrophoresis of nucleic acids as it had a high buffer capacity. About 108 g of *tris*-(hydroxymethyl)-aminomethane, *tris*-base ($C_4H_{11}NO_3$, Sigma-Aldrich), 55 g of boric acid (H_3BO_3, Sigma-Aldrich) and 9.3 g of ethylenediaminetetraacetate, EDTA ($C_{10}H_{16}N_2O_8$, Sigma-Aldrich) were added with 1 L of double-distilled water to give rise to 10x of TBE buffer. The pH is 8.3 and required no adjustment. The working buffer of agarose gel electrophoresis for nucleic acid was 1x TBE.

(V) Preparation of crystal violet staining solution

50mg of crystal violet(Sigma-Aldrich, USA) was added to 25mL of methanol and 75 double-distilled water to give 0.05% w/v crystal violet staining solution. The staining solution was stored in dark and room temperature.

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