The Hong Kong Polytechnic University
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Study of expression of candidate genes located in chromosome 5p of human esophageal squamous cell carcinoma

by

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A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Philosophy

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

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Miss Sarwat Fatima

August 2005

ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is a human cancer that is characterized by a high mortality rate and geographic differences in incidence. Previous studies of comparative genomic hybridization (CGH) showed that chromosome 5p is frequently amplified in cell lines and primary tumors of ESCC which are of Hong Kong Chinese origin. In the present study, we investigated the expression level of two novel genes located in 5p15.2 and are 5' upstream to delta catenin gene, named as JS-1 and JS-2, in cell lines and primary tumors of ESCC and also studied the transforming capacity of the two genes in normal cells. ESCC cell lines and patient cases with their matched non-tumor epithelial tissues were analyzed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for the overexpression of JS-1 and JS-2. The transforming capacity of JS-1 and JS-2 was also investigated by transfecting NIH 3T3 cells with their full coding sequences cloned into the expression vector pcDNA3.1(-) and followed by the study of foci formation under confluence growth and soft agar assay for investigating the anchorage independent growth property of the transfected cells. The JS-1 transfected cells with overexpression were also assessed for tumorigenicity in athymic nude

mice. Fourty-five percent (5/11) and 18% (2/11) of the ESCC cell lines showed overexpression of JS-1 and JS-2 respectively, while 56% (15/27) and 14% (3/22) of primary ESCC cases showed overexpression of JS-1 and JS-2 respectively. JS-1 overexpression was found in 26% (7/27) of patient cases with stage II tumors, 18% (5/27) cases with stage III tumors, 7% (2/27) cases with stage IV tumors and 4% (1/27) case with dysplasia, whereas JS-2 was only overexpressed in a case with dysplastic lesion (1/22; 4.5%) and in 9% (2/22) of patients with stage III tumors. Overexpression of JS-1 in NIH-3T3 cells also caused foci formation in confluence growth and colony formation in soft agar but not for JS-2. Subcutaneous tumor was formed in all nude mice tested when NIH 3T3 cells overexpressing JS-1 were injected subcutaneously into five athymic nude mice. Our results thus indicate that the frequent overexpression of JS-1 in ESCC and its transforming capacity in normal cells may play a critical role in the molecular pathogenesis of ESCC. The present study also forms the ground work on the further identification of novel mechanisms of molecular carcinogenesis in ESCC and other cancers.

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LIST OF ABBREVIATIONS

µg	:	microgram
µl	:	microlitre
a.a	:	amino acid
ATCC	:	American Type Culture Collection
bp	:	base pair
cDNA	:	complementary deoxyribonucliec acid
DMEM	:	Dulbecco's modified Eagle's medium
DMSO	:	dimethyl sulfoxide
DNA	:	deoxyribonucliec acid
dNTP	:	deoxyribonucleoside triphosphate
EDTA	:	ethylenediaminetetraacetic acid
e.g.	:	example
et al.,	:	et alia
ESCC	:	esophageal squamous cell carcinoma
EADC	:	esophageal adenocarcinoma
FBS	:	fetal bovine serum
g	:	gram/gravity
GAPDH	:	glyceryl aldehyde-3 phosphate dehydrogenase
H&E	:	hematoxylin and eosin
hr	:	hour
IPTG	:	isopropyl-β-D-thiogalactopyranoside
kb	:	kilobase
KCl	:	potassium chloride

KFSM	:	keratinocyte serum-free complete medium
LB	:	luria-bertani medium
$MgCl_2$	:	magnesium chloride
ml	:	millimeter
mM	:	millimolar
mRNA	:	messenger RNA
MTC panels	:	multiple tissue cDNA panels
MuLV	:	murine leukemia virus
no.	:	number
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
RACE	:	rapid amplification of cDNA ends
RNA	:	ribonucleic acid
RT-PCR	:	reverse transcriptase polymerase chain reaction
SCC	:	squamous cell carcinoma
SCC(M)	:	squamous cell carcinoma moderately differentiated type
SCC(P)	:	squamous cell carcinoma poorly differentiated type
SCC(W)	:	squamous cell carcinoma well differentiated type
TBE	:	tris-borate
U	:	units
UV	:	ultra violet

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Anatomy of human esophagus

1.1.1 Gross anatomy of the esophagus

The esophagus is a muscular tube that extends from the pharynx to the stomach. The length of the esophagus is defined anatomically as the distance between the cricoid cartilage and the gastric orifice. In adults, it ranges from 22cm to 28cm, of which 2cm to 6cm are located in the abdomen. It serves to transport masticated food. The esophagus descends through three compartments: the neck, the chest, and the abdomen. This progression has led to its division into cervical, thoracic and abdominal segments.

1.1.1.1 Cervical esophagus

Cervical esophagus begins at the lower end of the pharynx and extends to the thoracic inlet. It is 18cm from the upper incisors.

1.1.1.2 Thoracic esophagus

This is further divided into 3 parts. The upperthoracic portion extends from thoracic inlet to level of tracheal bifurcation. It is 18cm-23cm from upper incisors. The mid thoracic portion extends from the tracheal bifuraction midway to gastroesophageal junction. It is 24cm-32cm from upper incisors. The lower thoracic portion extends from midway between tracheal bifurcation and gastroesophageal junction, including abdominal esophagus. It is 32cm-40cm from upper incisors.

1.1.2 Histology of esophagus

Only the final portion of abdominal esophagus is lined with columnar epithelium, the rest of the epithelium within the esophagus is squamous. In addition, there are scattered glands within the esophageal submucosa which are lined with columnar epithelium. The submucosa layer is loosely attached to the mucosa, and this anatomical plane is quite easily disrupted. In contrast to the remainder of the gastrointestinal tract, the esophagus has no true serosal covering to limit distensibility. The mucosa of the esophagus is composed of three layers: epithelium, lamina propria and muscularis mucosae (Figure 1.1). The epithelium

lining of the esophagus is stratified, non-keratinized squamous which is very thick (300µm). The lamina propria is a loose connective tissue that sends papillae into the epithelium. It contains occasional lymphoid nodules. The muscular muscosae consists of a thick layer of smooth muscle which is sparse at the beginning, longitudinal in the middle and plexiform below. This layer replaces an elastic layer of the pharynx.

The submucosa of the esophagus is 3-700µm thick and home to coarse elastic fibers, accumulation of lymphoid tissue, large blood vessels, nerves and mucous glands. Muscularis propria: inner circular and outer longitudinal layers; proximally includes skeletal muscle from cricopharyngeus. Tunica adventitia (instead of serosa) is the outermost layer and comprises of loose connective tissue rich in elastin to allow for distension during swallowing and contains longitudinally oriented blood vessels, lymphatics and nerves.

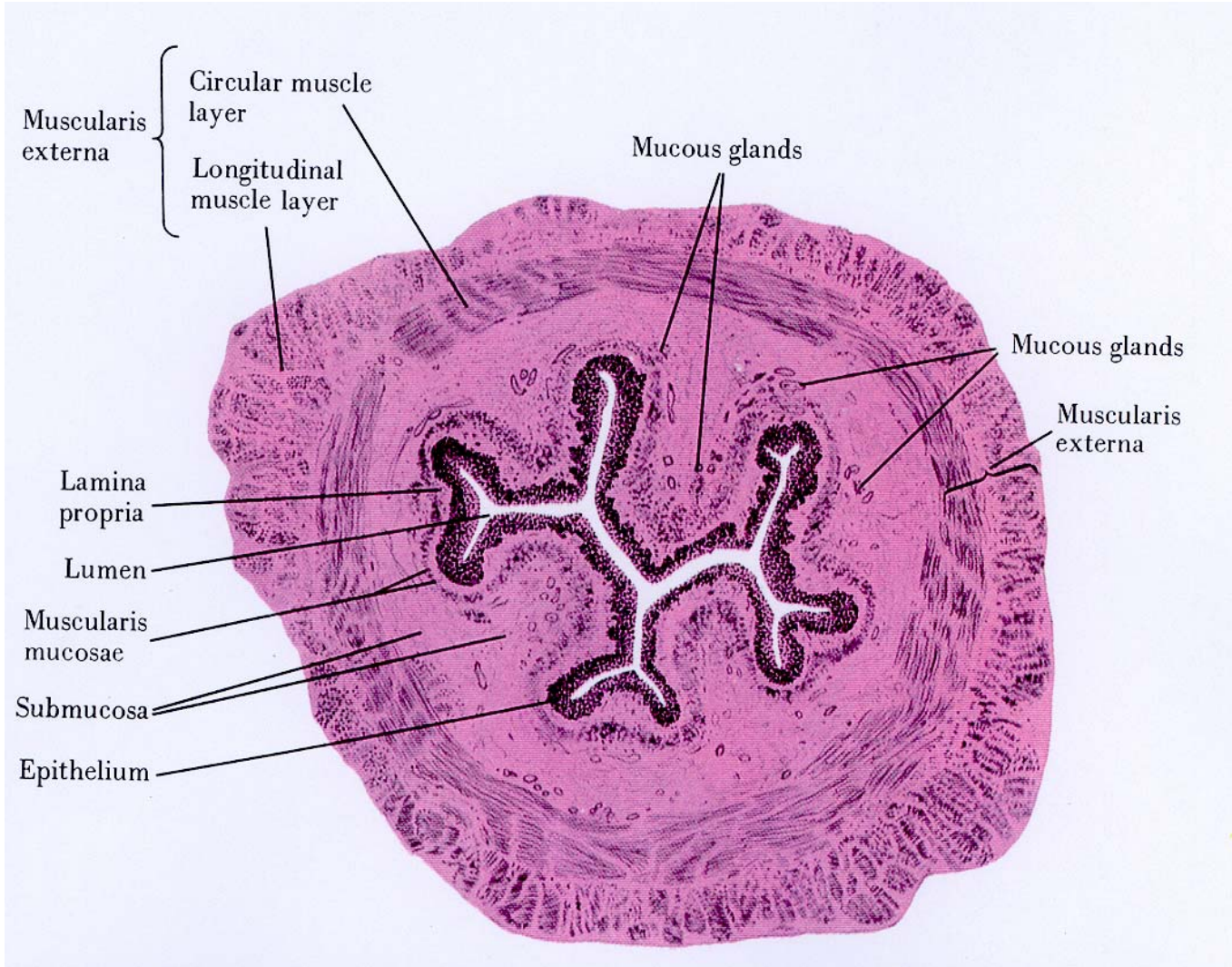


Figure 1.1 A cross-section of the human esophagus. The muscularis mucosa is relatively thick, thereby readily marking the extent of the mucosa. Mucosa glands are in the submucosa: their ducts which empty into the lumen of the esophagus, have not been included in the plane of the section. It is possible to distinguish the circularly arranged muscle fibres from those which are longitudinally disposed (and therefore seen in the cross-section). Adopted from Ross H. Micheal and Romrell J. Lynn. "Digestive system II: Esophagus and Gastrointestinal Tract". In *Histology: a text and atlas,* edited by Ross H Micheal and Romrell J Lynn. Baltimore: Williams & Wilkins, 1989, p. 423.

1.2 Esophageal squamous cell carcinoma

ESCC originates in the squamous epithelium and is prevalent in the mid-esophagus. A smaller number of tumors do arise in the distal and upper esophagus. ESCC accounts for 90% of all esophageal cancers worldwide (Stoner GD and Gupta A, 1995). The main precursor lesion of ESCC is epithelial dysplasia. Under a microscope, these lesions represent an accumulation of atypical cells with nuclear hyperchromasia, abnormally clumped chromatin and loss of polarity (Stoner GD and Gupta A, 1995). There is evidence that ESCC may develop from mild to severe dysplasia, carcinoma *in situ* and finally invasive carcinoma (Kuwano *et al.*, 1993 and Anani *et al.*, 1991).

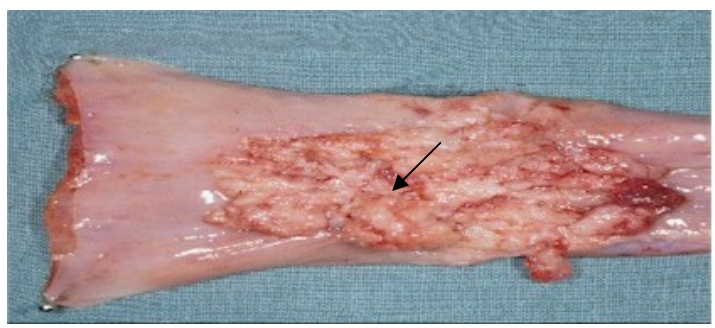
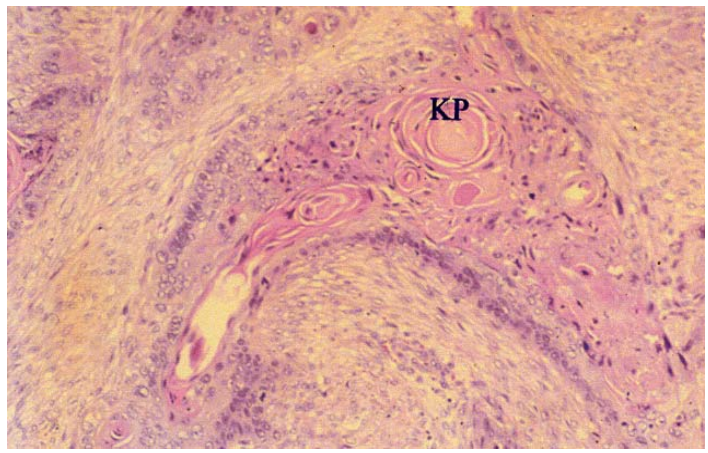


Figure 1.2 The gross appearance of ESCC (arrow) on mucosal surface of esophagus.

1.2.1 Histological grade

Squamous cell carcinomas are designated as well, moderately and poorly differentiated. Well differentiated tumors exhibit orderly stratification, obvious cellular bridges, and keratin pearl formation. In contrast, poorly differentiated squamous cell carcinomas are noted for their high nucleus-cytoplasmic ratio. Keratin pearl formation is absent but individual cell keratinsation may be present. They also lack intercellular bridges. Moderately differentiated tumors fall somewhere in between (Figure 1.3).

a)



b)

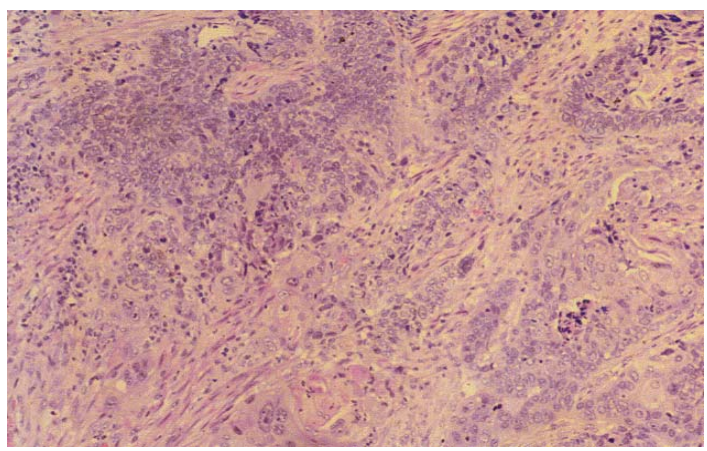


Figure 1.3 Histopathological features of ESCC of (a) well-differentiated type (x100) and (b) poorly differentiated type (x200). Well-differentiated ESCC is characterized by the presence of keratin pearls (KP). The poorly differentiated tumor shows lack of resemblance to normal pickle cells and has a high nucleus-cytoplasmic ratio. Adopted from Wheater P.R., Burkitt G, Stevens A, and Lowe J.S. "Alimentary system". In: *Basic histopathology*, edited by Wheater P.R., Burkitt G., Stevens A and Lowe James. New York: Churchill Livingstone, 1991, p. 115-133.

1.2.2 Tumor staging

Tumor staging assesses severity of a tumor. The TNM system is one of the most commonly used staging systems. It has been accepted by the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC). The TNM system (Table 1) is based on the extent of the tumor **(T)**, the extent of spread to the lymph nodes **(N)**, and the presence of metastasis **(M)**.

Table 1. TNM classification of esophageal carcinoma (adopted from Wobst *et al.,* 1998).

Stage	Description
T	Primary tumor
TX	The primary tumor cannot be assessed
T0	No evidence of a primary tumor
Tis	Carcinoma *in situ*
T1	Tumor invades mucosa or submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades adventitia
T4	Tumor invades adjacent structures
N	Regional lymphodes
NX	The regional lymph nodes cannot be assessed
N0	No regional nodal metastases
N1	Regional lymph node metastases
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

1.2.3 Prognosis

The most important prognostic factor for ESCC is the extent of the disease at the time of diagnosis. Patients diagnosed with stage I tumor ($T_1N_0M_0$) have the highest, 50-80%, five year survival rate after surgical resection. However, less than 1% of patients are diagnosed with stage I tumor. The main reason for poor prognosis for esophageal carcinoma is that most tumors are asymptomatic and go undetected until they are unresectable (Enzinger PC and Mayer RJ, 2003).

Another reason for poor prognosis is partly due to the fact that ECCC with lymph node metastasis often encounters rapid reoccurrences. ESCC patients with lymph node metastasis have a significantly poorer five year survival rate than that of patients without lymph node metastasis; therefore lymph node metastasis is considered an important prognostic factor for ESCC (Nakajima *et al.,* 2002). Surgical resection offers a better chance of cure if the disease is diagnosed early, but tumor growth is rapid once the neoplasm involves the submucosal layer (Haruma *et al.,* 1991). The most important factor in reducing the esophageal carcinoma mortality is new strategies for early diagnosis to treat the curable.

1.2.4 Epidemiology

The incidence of esophageal cancer varies considerably with geographic location and also, to some extent, among ethnic groups within a common area, suggesting a predominant role for external environmental factors (Koshy *et al.,* 2004).

Some of the highest rates of ESCC occur in Linxian region of China, and northern Iran, where incidence rate exceeds 100 in 100,000 individuals. These two countries are part of what is known as the "Esophageal Cancer Belt of South-Central Asia" an area that stretches from the Caucasian mountains across northern Iran, Afghanistan, Kazakhestan, Uzbekistan and Turkamanistan, into northern China. In the past few decades ESCC has also reached endemic levels in Sub-Saharen Africa. Its incidence remains low, as in the Ivory Coast, Mali and the Gambia. However, in other African countries, the incidence rate has risen considerably, especially in city populations, as in Durban, South Africa, in Kyadondo, Uganda, and in Harare, Zimbabwe (Walker *et al.,* 2002).

According to the Hong Kong cancer registry, esophageal cancer was ranked as the 10th most common cancer in Hong Kong, accounting for more than 2% of all cancer cases in 2002. It also ranked as the 7th most fatal cancer in 2003. The

disease is more common in males and the male to female ratio is about 4.6 to 1. ESCC accounts for 89% of all esophageal cancers in China including Hong Kong and since 1980's, esophageal cancers in Hong Kong have remained constant (Law S and Wong J, 2002). In contrast, EADC is more predominant in Western countries (Devesa *et al.,* 1998).

1.2.5 Etiology

1.2.5.1 Alcohol and tobacco related esophageal cancer

The etiology of esophageal cancer remains unclear. There are several factors that have been found to be associated with increased risks of ESCC. Alcohol and tobacco have been identified as main risk factors in North and South America, Europe and South Africa. A direct relationship has been found between the amount of alcohol and cigarettes consumed daily. One study found that certain ways of tobacco consumption carry more risks than others. For example, the risk may be greater among pipe smokers and smokers of hand rolled cigarettes than among smokers of commercial cigarettes (Tuyns AJ and Esteve J, 1983). Italians who smoke high-tar cigarettes are at higher risk than those who smoke middle or low tar cigarettes (La Vecchia *et al.,* 1986). A study in Italy also suggested that

the component of alcohol that determines the risk of ESCC is ethanol (Bosetti *et al.,* 2000).

1.2.5.2 Non-alcohol and tobacco related esophageal cancer

Risk factors for ESCC in endemic regions of Iran and China are still not known. But unlike other regions mentioned above, alcohol consumption and tobacco are not regarded as high risk factors in these populations. In Iran, opium smoking and chewing of opium pipe scrapings may play an important causal role in the risk of ESCC development (Cook *et al.,* 1979). In China, N-nitrosocompounds have been implicated as important risk factors (Lin *et al.,* 2002). In the Chaoshan region of China, a strong dose-response relationship was found between fermented fish sauce and high risk of ESCC in the population. Intake of fresh fruits and vegetables showed a decreased risk. However, pickled vegetables, was found to be positively associated with the cancer in Hong Kong Chinese patients and not with those from China (Li K and Yu P, 2003).

1.2.5.3 Cultural, dietary and environmental considerations

Cultural practices of a particular region may also play a role in the risk of ESCC. Very hot maté drinking has been significantly associated with ESCC in South

America (Castellsague *et al.,* 2000). Mate is drunk through a metal straw and is swallowed very quickly. The risk factor could either be due to the components of mate´ or due to the temperature. In China, drinking "of burning hot beverages" has been implicated for esophagitis (Wahrendorf *et al.,* 1989). A case-control study in Hong Kong suggested that the consumption of hot drinks and soups accounted for 14% of esophageal cancers in the population (Cheng *et al.,* 1992). In Iran drinking hot tea has also been suspected as a risk factor (Ghavamzadeh *et al.,* 2001).

In some areas, bread baked weakly and then eaten moldy, has also been implicated as a risk factor. In China, it is common practice in some regions to eat dried persimmons, a rough food that injures the esophageal mucosa. Also, in South Africa, high content of maize in locally brewed beer is also a risk factor and the risk does not rest solely on the quantity of alcohol consumed (Segal *et al.,* 1988).

1.2.5.4 Nutritional factors

A diet poor in fresh fruits, vegetables and dairy products has long been proposed as augmenting the risk of esophageal cancer (Li K and Yu P, 2003;

Ghavamzadeh *et al.,* 2001; Victora *et al.,* 1987). Case-control studies in Italy and Iran have found an inverse relationship between, vitamins A, C, E and niacin and the risk of ESCC (Franceschi *et al.,* 2000; Siassi *et al.,* 2000). Increased risk of ESCC has also been associated with Plummer Vinson Syndrome, which is caused by a lack of iron and vitamins (Ribeiro *et al.,* 1996).

1.2.5.5 Associated diseases

Fourty percent of people with Tylosis go on to develop ESCC. Thirthy percent of people with Achalasia, and 10% of people with Plummer-Vinson syndrome are also at risk of developing ESCC. Chronic mucosal damage has been proposed as the likely culprit. Other diseases include esophageal webs, celiac disease, head and neck cancer and human papilloma virus (HPV) infection (Lukanich JM, 2003).

1.2.6 Symptoms

Esophageal squamous dysplasia (ESD) is the most common precursor lesion for ESCC and has been observed in endemic regions including China, Iran, South Africa and Northwestern France. Randomly selected individuals from these high-risk population areas have an incidence of ESD ranging from 5% to 40%.

However, the incidence of ESD has ranged from 3.6% to 6% in populations at low-risk from ESCC (Dry SM and Lewin KJ, 2002).

Patients with early esophageal cancer maybe also hoarse, have hiccups and elevated calcium levels. Most patients with esophageal cancer have dysphagia at the time of diagnosis. Painful swallowing is usually a symptom of a large tumor obstructing the opening of the esophagus. It may lead to weight loss, physical wasting and malnutrition (Dr. Smith J.F. Medical Library. Esophageal cancer: symptoms. Retrieved 28th December 2004 <http://www.chclibrary.org/micromed/00047310.html>).

1.2.7 Therapy

Surgical resection remains the treatment of choice for ESCC patients but the results are disappointing (Wilson *et al.*, 2000). Post operative chemotherapy, radiotherapy and chemo-radiotherapy have also failed to improve prognosis (Ando *et al.*, 1997; Group *et al.*, 1993; Teniere *et al.*, 1991). At present, the strategy that shows the most potential is preoperative chemotherapy followed by surgery (Yano *et al.*, 2005). A phase III trial from the United States (Radiation Therapy Oncology Group trial 85-01) indicated that chemo-radiotherapy is better

than radiotherapy alone and led to the inclusion of primary chemo-radiotherapy as a standard treatment of care by the United States (Cooper *et al.,* 1999). Cyclooxygenase-2 (COX-2) has been shown to be overexpressed in ESCC (Kuo *et al.,* 2003; Zimmermann *et al.,* 1999) and recently Takatori *et al.,* (2005) have reported that chemo-radiotherapy was more effective in patients with low COX-2 mRNA expression than in those with high expression. Zimmermann *et al*., (1999) also proposed COX-2 inhibitors for therapy in ESCC following reduction in proliferation and induction of apoptosis in an esophageal cancer cell line treated with COX-2 inhibitors.

1.3 Molecular aspects of ESCC

1.3.1 Oncogenes

c-myc

The myc oncogene encodes p62, a phosphoprotein that forms heterodimers with MAX and is required for cellular proliferation and differentiation (Lam AK, 2000). Miyazaki *et al.,* (1992) reported *c-myc* amplification and overexpression in 20% of ESCC and in adjacent-normal esophageal mucosa. *c-myc* amplification has been reported more frequently in advanced stages of ESCC than in early stages and also its amplification has not been associated with overall survival of ESCC patients treated by surgery or by multimodal therapy (Bitzer *et al.,* 2003).

Cyclin D1

Cyclin D1 controls progression from G1/S check-point in the cell cycle. Gene amplification and over expression of cyclin D1 have been reported in ESCC cell lines and 22-73% of ESCC tumors (Nagasawa *et al.,* 2001; Shamma *et al.,* 2000; Nakagawa *et al.,* 1995; Morgan *et al.,* 1999). Overexpression of cyclin D1 confers increased tumor cell proliferative activity, high chance of lymphatic

vessel invasion and metastasis to lymph nodes (Nagasawa *et al.,* 2001) and could be an important prognostic marker for ESCC. Cyclin D1 overexpression in ESCC patients has also been correlated to poor response to chemotherapy (Sarbia *et al.,* 1999; Nagasawa *et al.,* 2001) and poor overall survival in ESCC patients who have undergone surgically treated ESCC (Sarbia *et al.,* 1999).

Mdm2 (Murine double minute 2)

Mdm2 gene product, a 90kDa protein, can inactivate wild-type p53 protein and Rb protein. Morgan *et al.,* (1999) and Shimada *et al.,* (1999) reported mdm2 gene amplification in 22% and 19% of esophageal cancers respectively. Ikeguchi *et al.,* (2002) correlated short patient survival only in ESCC patients who underwent postoperative adjuvant chemotherapy and found no correlation between mdm2 overexpression and patients undergoing adjuvant therapy.

Growth factors

Growth factors allow cells in the resting phase (G0) to proceed through the cell cycle. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have been reported to function as autocrine growth factors for ESCC when overexpressed in ESCC cell lines (Wong *et al.,* 1994; Yoshida *et al.,* 1990).

Growth factor receptors

Epidermal growth factor receptors exhibit tyrosine kinase activity and serve as receptors for epidermal growth factors (Takaoka *et al.,* 2004). There are four members of this family, namely erbB-1, erbB-2, erbB-3 and erB-4. However, only erbB-1 and erbB-2 have been studied in ESCC.

erbB-1

erbB-1 overexpression and/or amplification have been reported in primary ESCC and its overexpression may also be correlated to poor prognosis (Inada *et al* ., 1999; Suzuki *et al.,* 1997; Kitagawa *et al.,* 1996; Itakura *et al.,* 1994). Overexpression of erbB-1 was also associated with minor response to chemoradiotherapy (Miyazono *et al.,* 2004).

erbB-2

Overexpression of erbB-2 is not common in ESCC (Shiga *et al.,* 1993). However, Akamatsu *et al.,* (2003) reported erbB-2 overexpression as an indicator of chemoresistance in ESCC.

Signal transducers

Ras proteins resemble G proteins and serve as signal transducers and regulate cell growth and differentiation (Campbell *et al.,* 2004). No mutation in ras gene has been reported in ESCC (Galiana *et al.,* 1993) but the overexpression of p21, a product of ras gene, has been reported in ESCC and it may be related to higher proliferative condition of cancer cells in comparison to normal cells (Lam *et al.,* 1995; Ruol *et al.,* 1990).

1.3.2 Tumor suppressor genes

p53

Mutations of p53 play an important role in the development of many human cancers. p53 mutations in ESCC varies geographically. Audrezet *et al.,* (1993) and Tamura *et al.,* (1992) reported p53 mutations in 84% and 38% of ESCCs from France and Japan respectively. Gamieldien *et al.,* (1998) and Gates *et al.,* (1994) reported that 67% and 17% of esophageal tumors were associated with p53 mutations in South Carolina and South Africa. p53 mutations also vary within a geographic region. Cao *et al.,* (2004) reported higher p53 mutation in a high risk area of China compared to a lower risk area. In ESCC, most p53 mutations exist within exons 5 to 8 and are point mutations. p53 mutations with

G → T transversions have been associated with cigarette smoke and alfatoxin (Montesano *et al.,* 1996). p53 protein accumulation has been detected in normal squamous epithelium and its expression increases in basal cell hyperplasia, preneoplastic lesions and squamous cell carcinomas suggesting that p53 mutations occur in early stages of ESCC. The build up of p53 protein could represent a malfunction protein due to mutation of the p53 gene or it could also represent accumulation of wild type p53 protein in response to DNA damage (Gao *et al.,* 1994; Hainaut P, 1995). Nuclear accumulation of p53 has been reported to show a significantly lower survival rate in ESCC patients than in ESCC patients with no accumulation (Uchino *et al.,* 1996; Shimaya *et al.,* 1993). There is conflicting data on whether mutation in p53 gene determines response to chemotherapy/radiotherapy. Lam *et al.,* (1997b) and Ito *et al.,* (2001) have reported no correlation between p53 mutation and response to chemotherapy/radiotherapy. However, Ribeiro *et al.,* (1998) reported that p53 genotyping may identify patients who respond well to chemo-radiotherapy.

p21

p21 gene is a tumor suppressor gene which is up-regulated by wild-type p53 gene at the transcriptional level (Hamada *et al.,* 2004). In China and Japan, p21

expression has been detected in 27-85% of ESCC patients (Shimoyama *et al.*, 1998; Yang *et al.*, 1997) and in Hong Kong 70% of ESCC patients were found to have p21 expression (Lam *et al.*, 1999). The correlation between p21 expression and prognosis remains controversial. A few studies have reported reduced expression of p21 to poorer prognosis in ESCC (Nita *et al.*, 1999; Natsugoe *et al.*, 1999) while some have reported overexpression of p21 to poorer prognosis (Lam *et al.*, 1999; Sarbia *et al.*, 1998a). Nakajima *et al.*, (2004) reported p21 expression as a good indicator of ESCC to CPT-11, a therapeutic agent against ESCC. CPT was ineffective in ESCC patients showing low expression of p21.

p16 and p15

p16 and p15 tumor suppressor genes are mapped to chromosome 9p21-22 and loss of heterozygosity on chromosome 9 is a common occurrence in most cancers. p16 and p15 genes control the progression of the cell cycle from the G1 to S phase (Tokugawa *et al.*, 2002). p15 is a hotspot for homozygous deletion whereas hypermethylation is a primary target for p16 (Xing *et al.*, 1999a). Takeuchi *et al.*, (2001) reported that altered expression of p16 is associated with lymph node metastasis in ESCC. Tokugawa *et al.*, (2002) reported that p16 hypermethylation is not restricted to advanced ESCC but also occurs in precursor

lesions and background normal-looking epithelial. In esophageal cancer, mutation of p16 varies from 0% to 52% (Hu *et al.,* 2004). The differences could be due to differences in population size, ethnicity and geography. Chan *et al.,* (1997) reported only 12% mutations in Hong Kong Chinese where as Xing *et al.,* (1999b) reported no mutations in 34 cases from Linxian, an area in China with one of the highest incidence of ESCC. p15 alterations have not been studied as frequently as p16 alterations in ESCC. p15 mutation was found in 3% of Japanese ESCC samples (Suzuki *et al.,* 1995) and in 45% of Chinese ESCC samples (Xing *et al.,* 1999b). Further investigations into p15 alterations are required to understand its role in ESCC.

Retinoblastoma (Rb)

Rb gene product is a nuclear phosphoprotein that influences the cell cycle. The nonphosphorylated Rb prevents cell proliferation and must be suppressed in order to pass through the cell cycle. The phosphorylated form of Rb (by CDK/cyclin D1) releases constraint on cell growth (Lam AK, 2000). Frequent mutation and loss of Rb gene have been reported in ESCC (Ikeguchi *et al.,* 2001a; Huang *et al.,* 1993). The expression of Rb is commonly associated with cyclin D1 overexpression (Ishikawa *et al.,* 1998) and its been reported that cyclin D1

overexpression may suppress Rb function and that collective analysis of cyclin D1 and Rb may possibly be helpful in prognosis of ESCC patients (Ikeguchi *et al.,* 2001a; Jiang *et al.,* 1993).

1.3.3 Metastasis related genes

nm23 was originally identified as an anti-metastatic gene whose expression is inversely correlated to metastatic potential in murine melanoma cell lines (Steeg *et al.,* 1988). Patel *et al.,* (1997) reported loss of nm23 in 71% of ESCC samples and correlated the loss to reduction in survival rate. Reduced expression of nm23 has also been reported to increase the sensitivity of ESCC patients to cisplatin treatment. Thus, nm23 expression maybe a good predictor of sensitivity to cisplatin chemotherapy treatment in ESCC patients (Wang *et al.,* 2004; Iizuka *et al.,* 2000).

Two additional categories of metastasis-related proteins studied in ESCC include cell-adhesion molecules and enzymes involved in degradation of extra-cellular matrix.

In epithelial cells, E-cadherin represents a key molecule in the establishment and stabilization of cellular junction. It regulates cell-adhesion by interacting with E-cadherin molecules on opposing cell surfaces (Hajra *et al.*, 2002, Aberle *et al.*, 1996). Abnormal expression of E-cadherin has been correlated to distant metastases in ESCC (Jian *et al,* 1997). Relationship between E-cadherin expression and survival remains controversial. Inada *et al.*, (1999) reported that a five year survival rate of patients with ESCC ranged from 87.8% in normal E-cadherin expression to 19.1% in absent expression of E-cadherin. Nakashini *et al.*, (1997) demonstrated no significant correlation between survival and E-cadherin expression. Takayama *et al.*, (2003) investigated the differences between the expression of adhesion molecules (E-cadherin, β-catenin, CD44, CD44-v6 and Integrin β1) in metastatic lymph nodes and their primary tumor. They reported that a reduced expression of E-cadherin, beta-catenin and CD44-v6 in the metastatic lymph nodes correlated with an increased number of lymph node metastases in ESCC.

Degradation of extracellular matrix (ECM) by matrix metalloproteinases (MMPs) plays a part in invasion and metastasis (Woessner JF, 1991). The MMPs family consist of collagenases, galatinases, and stromelysins. Expression of either of these types of MMP in ESCC samples has been associated with degree of tumor invasiveness, lymph node metastasis, and poor prognosis (Gu *et al.*, 2005; Sharma *et al.*, 2004; Yamashita *et al.*, 2004).

1.3.4 Apoptosis related genes

Apoptosis plays a key role in the pathogenesis, aggressiveness, and therapy responsiveness to cancer. Bcl-2 family proteins are regulators of apoptosis and members of this family that have been studied extensively in ESCC are bcl-X and bcl-2 proteins. These two proteins play the role of anti-apoptosis and have an inverse expression pattern. Takayama *et al.*, (2001) reported that as the depth of ESCC tumor advanced, bcl-X expression increased and bcl-2 expression decreased. Sarbia *et al.*, (1998b) reported that bcl-X expression showed more frequent response to chemotherapy. Bcl-X was also closely associated with lymph node metastasis and prognosis (Takayama *et al.*, 2001). On the other hand, reduced expression of bcl-2 expression has been reported in ESCC progression and it is expressed in early stage ESCC (Takayama *et al.*, 2001;

Ohbu *et al.,* 1997; Parenti *et al.,*1997; Sarbia *et al.,* 1996). Bcl-2 has no affect on overall survival (Sarbia *et al.,* 1996). Bax is another member of blc-2 family of proteins and it promotes apoptosis. Kim *et al.,* (2001) transfected a low sensitivity esophageal carcinoma cell line with bax gene and reported an elevated response to radiation sensitivity associated with increased apoptosis following irradiation. Thus bax gene may determine response to irradiation therapy. Ikeguchi *et al.,* (2001b) also reported similar findings that bax expression correlated with good prognosis in patients who underwent postoperative chemo-radiotherapy.

1.3.5 Loss of heterozygosity (LOH)

Microsatellite marker LOH studies have shown that allelic losses on chromosomes 1p, 3p, 9, 11q, 13q and 17 are common in ESCC. Allelic loss of chromosome 9p21-22 has been reported in 70% of ESCC. Tumor suppressor genes residing in this region are CDKN2A and CDKN2B, encoding p16 and p15 respectively. CDKN2A and CDKN2B inhibit cyclin dependent kinase 4 (CDK4) and CDK6 and control cellular proliferation by preventing entry into S phase of the cell cycle (Hu *et al.,* 2004). Other commonly deleted regions in chromosome 9 include 9p23-22, 9q13-22.3, and 9q34 (Lichun *et al.,* 2004). A novel tumor

suppressor gene, DEC1, was identified in 9q32 region and it showed reduced expression in 62% of ESCC cell lines and 53% of primary ESCC (Nishiwaki *et al.,* 2000). Yang *et al.,* (2005) also showed 74% LOH in the DEC1 region of primary ESCC tumors. Allelic loss on chromosome 17p13 is frequent in ESCC, which includes the region where p53 resides. However, a good correlation between allelic loss of 17p and mutation of p53 has not been found, suggesting that unknown tumor suppressor genes near p53 may play a role in the development of ESCC (Huang *et al.,* 2000). Region 17q is where the TOC gene resides and the frequent loss of heterozygosity in this region is worthy of note. Tylosis is an autosomal dominant trait characterized by *hyperkeratosis palmaris et plantaris* that has been associated with high risk of ESCC (Risk *et al.,* 1999). Allelic loses have also been reported on other chromosomal regions including 3q21.3, 9q31 and 13q. Chromosome region 3q21.3 contains a novel tumor suppressor gene, DLC1, whose loss of functional transcripts and increase in nonfunctional RNA transcripts was reported in 30% of ESCC (Daigo *et al.,* 1999). Another novel gene, ING1, has been mapped to 13q33-44 and has low level of expression of ING1 protein (Chen *et al.,* 2001).

1.3.6 Methylation

Several ESCC related genes have been reported to be silenced by methylation. These genes include E-cadherin (Takeno *et al.,* 2004), $p16^{INK4a}$ (Kwong *et al.,* 2004a), human leukocyte antigen (HLA) class I genes, $p15^{INK4b}$,$p14^{ARF}$ and hMLH1 (Nie *et al.,* 2002). Hypermethylation signatures of genes could be a useful tool for diagnosis.

1.4 The human genome project

The Human Genome Project (HGP) was an international collaboration that began in 1990 with aim of mapping all the genes and to determine the nucleic acid sequence of the human DNA. The near-complete sequence was published in 2004 and the entire human genome sequence is available on the World Wide Web for everyone.

The most immediate application of the human genome is to speed up the identification of genes whose malfunction causes human diseases. It will allow development of new diagnostic tools based on understanding genetic variations that underlie cancers. The completion of the human genome project (HGP) has resulted in a vast amount of DNA sequences in the GenBank and this means there are hundreds of novel genes whose roles in living cells are uncertain. The notion that there are several genes whose functions remain to be elucidated tells us that there are loopholes in our knowledge of basic biology. Thus it is important to decipher the functions of such hypothetical genes. In the present study we have studied the expression of two hypothetical genes located on chromosome 5p15.2 and that are upstream to delta catenin gene.

One strategy to identify human disease genes is based on analyzing gene expression (Austin CP, 2004). This is the strategy that I have utilized in my project. The presence of mRNA in pathological and not in normal tissue suggests that the gene could be involved in a disease. The caveat for this type of study is that it demonstrates correlation and not causation. With the help of DNA microarray, gene expression alterations in diseases are also being used in clinical practice. The first large clinical trial has begun in Europe to treat patients on the basis of their gene expression profiles (Branca M, 2003). Gene expression profiles were also used to distinct subgroups of diffuse large B cell lymphoma (Wright *et al.*, 2003).

1.5 Clonal evolution of tumor cell populations

Cancer is a disease of mutation and natural selection. Nowell PC (1976) was the first to suggest the concept of clonal evolution of cancer. First, one daughter cell either inherits or acquires a cancer-promoting mutation believed to be from a carcinogen. Such a change leads to selectable growth advantage and allows its progeny to expand as a neoplastic clone. Neoplastic proliferation then proceeds either immediately or after a latent period under the selection of micro environmental pressure generated by the host. Most mutant cells will be eliminated as a result of metabolic disadvantages or they will be destroyed by the host immune system. However, there will be one such mutant that will acquire additionally selective advantage over the original tumor cells and normal cells. This mutant then becomes the new precursor for a predominant subpopulation with enhanced survival and malignant characteristics. The defect is passed on to subsequent generations. Later on, a descendant acquires a second mutation and a later descendant acquires a third. Over time, sublines with increasingly abnormal phenotypic and genotypical characteristics will be selected by evolutionary pressure. Once a cell accumulates enough mutations it becomes malignant. Hanahan and Weinberg (2000) described six trademark alterations a normal cell

undergoes to acquire malignancy: (a) self-sufficiency in growth signals; (b) insensitivity to growth-inhibitory (antigrowth) signals; (c) evasion of programmed cell death (apoptosis); (d) limitless replicative potential; (e) sustained angiogenesis; and (f) tissue invasion and metastasis.

Cancer cells can become malignant in different ways. Mutation of particular genes may only be acquired in a small subset of histologically identical tumors; mutations of oncogenes or tumor suppressor genes may occur at the initial stages of a particular tumor and late in others; resistance to apoptosis, unlimited replicative potential, sustained angiogenesis and other biological capabilities can be acquired at different times in the same cancer types and amongst different cancers (Hanahan D and Weinberg RA, 2000). The fact that a population of cells can be so heterogeneous in terms of their genetic mutations means that most treatments cannot eradicate all the cells. Furthermore, since each patient's cells acquire/inherit independent number and types of mutations, cancer cells in each patient is possibly unique. Consequently, cancer treatments cannot be generalized.

1.6 Genes studied in the present project

Genomic amplification of chromosome 5p has been reported in ESCC cell lines and tissue samples by several comparative genomic hybridization (CGH) studies (Kwong *et al.,* 2004b; Han *et al.,* 2002; Hu *et al.,* 2002; Wei *et al.,* 2002; Yen *et al.,* 2001; Tang *et al.,* 2001a; Tada *et al.,* 2000; Wang *et al.,* 1998). Furthermore, poor prognosis has been reported in ESCC patients with 5p chromosomal amplification (Ueno *et al.,* 2002). The genomic amplification of this region may suggest possible overexpression of gene(s) which may be relevant to the molecular carcinogenesis of ESCC.

This study investigates the expression of two novel genes, JS-1 and JS-2. These two genes have been predicted by the human genome project for their locations in chromosome 5p15.2 region and they are 5' upstream to a known delta catenin gene. Our collaborative group in the USA (Dr. Qun Lu from the University of North Carolina) is investigating the expression of delta catenin in ESCC. The overexpression of delta catenin has been implicated in a few cancers, e.g. gliomas (Shai *et al.,* 2003) and prostate cancer (Burger *et al.,* 2002) and this study will look into expression JS-1 and JS-2.

1.7 Aims and objectives of the thesis

The etiology of ESCC has been studied extensively and several oncogenes and tumor suppressor genes have also been reported to be closely related to the molecular pathogenesis of ESCC, such as *c-myc*, cyclin-D1, Rb and p53, but the complete picture of the molecular and genetic bases for the carcinogenesis of ESCC still remains largely unknown. The work of the Human Genome Project (HGP) has resulted in a vast amount of genomic sequences and the functional roles of the predicted genes in particular chromosomal regions remain to be elucidated. Thus, many more genes may be involved in the development of ESCC. This study aims to study two novel genes, named as JS-1 and JS-2, located on chromosome 5p, a region frequently amplified in ESCC. Detailed objectives of the thesis are summarized below.

1) To detect overexpression of two novel genes, JS-1 and JS-2, in ESCC cell lines and in ESCC specimens along with their corresponding non-tumor epithelial tissues.

2) To correlate the expression of JS-1 and JS-2 to clinicopathological

parameters to understand their significance in ESCC.

3) To study the transforming capacity of JS-1 and JS-2 *in vitro* by cloning their full-length coding regions into an expression vector followed by transfection into NIH 3T3 cells to monitor their cell growth, foci formation and anchorage-independent growth in soft agar.

4) To investigate the tumorigenicity of JS-1 *in vivo* by subcutaneously injecting NIH 3T3 cells overexpressing JS-1 gene into athymic nude mice to observe tumor formation.

5) To conduct bioinformatics analysis on JS-1 and JS-2 transcripts and proteins for the purpose of understanding their possible functions.

The overall results of the present study would add to our knowledge about the importance of differentially expressed genes in chromosome 5p region that could play a role in the molecular carcinogenesis of ESCC. The findings of the present study would also pave the way for future studies about the molecular pathogenesis of these novel genes in other cancers.

CHAPTER 2 MATERIALS AND METHODS

2.1 Samples used in the present study

2.1.1 Cell lines

Mouse fibroblast cell line, NIH 3T3, non-tumor esophageal epithelial cell line, NE3, and eleven ESCC cell lines including, SLMT-1 (Tang *et al.*, 2001a), HKESC-3 (Hu *et al.*, 2002) and KYSE 30, 70, 140, 150, 180, 410, 450, 510, 520, (Shimada *et al.*, 1992) were used in the present study. SLMT-1 and HKESC-3 cell lines were kindly provided by Professor Gopesh Srivastava of the Department of Pathology, The University of Hong Kong. The KYSE series of cell lines were purchased from DSMZ (Braunschweig, Germany). A HPV E6E7 immortalized non-tumor esophageal epithelial cell line (NE3) was kindly provided by Professor George S. W. Tsao of Department of Anatomy, The University of Hong Kong. NIH 3T3 cell line was purchased from the American Type Culture Collection (ATCC). The characteristics of the eleven ESCC cell lines used in this study are detailed in Table 2.

ESCC cell lines	Age	Sex	Histopathological type	Prior treatment on tumor
KYSE-30	64	M	SCC (W)	N.D.
KYSE-70	77	M	SCC (P)	N.D.
KYSE-140	54	M	SCC (M)	N.D.
KYSE-150	49	F	SCC (P)	Irradiation
KYSE-180	53	M	SCC (W)	N.D.
KYSE-410	51	M	SCC (P)	N.D.
KYSE-450	59	M	SCC (W)	N.D.
KYSE-510	67	F	SCC (W)	Irradiation, CDDP
KYSE-520	58	F	SCC (M)	N.D.
HKESC-3	74	M	SCC (M)	N.D.
SLMT-1	49	M	SCC (W)	N.D.

Table 2. Information on ESCC patients from whom the ESCC cell lines were established. SCC, squamous cell carcinoma; CDDP, cisplatin; (D), dysplasia; (W), well differentiated type of tumor; (M), moderately differentiated type of tumor; (P), poorly differentiated type of tumor; N.D., not done.

All ESCC cell lines and NIH 3T3 cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS), (Gibco). NE3 cell line was cultured in KSFM (keratinocyte serum-free complete medium) (Gibco). In addition to their respective culture mediums, all cell lines were maintained in 100µg/ml penicillin (Gibco) and 100units/ml of streptomycin (Gibco) and incubated at 37°C with 5% CO_2. When the cultures were confluent, they were washed with phosphate buffered saline (PBS) and treated with 0.05% trpsin (Gibco) in 0.05% EDTA and split in 1:3 ratio for subculture. Cell pellets for RNA extraction were obtained by spinning the cells following trypsinisation and discarding the supernatant. The cell pellets were stored in -70°C until used.

Liquid nitrogen stocks were prepared by freezing the cells in 10% dimethyl sulfoxide (DMSO), 70% of their respective medium and 20% FBS for 24 hours in -80°C followed by storage in liquid nitrogen.

2.1.2 ESCC tissue specimens

Twenty seven ESCC tissue specimens and their corresponding non-tumor tissues, at least 5cm away from tumors, used in the present study were randomly obtained by surgical resection from ESCC patients at the Queen Mary Hospital, Hong Kong between 1990-1998. All the selected ESCC specimens had more than 80% viable tumor cells as shown by histological examination. Tissue specimens were snap frozen in liquid nitrogen and stored in -70°C until used. Table 5 (in chapter 3) lists the clinicopathological features of the ESCC tissue specimens studied.

2.2 RNA extraction and reverse transcription

Total RNA was extracted from ESCC cell lines, frozen ESCC tumor tissues and their corresponding non-tumor tissues using TRIzol (Invitrogen)/chloroform method according to the manufacturer's protocol. The samples were homogenized in 1ml of TRIzol reagent (Gibco) and incubated for 5 minutes at room temperature. 200µl chloroform per ml of TRIzol reagent was added to the mixture. The tubes were shaken vigorously for 15 minutes and incubated at room temperature for 3 minutes. The upper aqueous phase was collected following centrifugation at 12,000g for 15 minutes. RNA was then precipitated from the aqueous phase by mixing with 500µl isopropyl alcohol and centrifugation at 12,000g for 10 minutes. The supernatant was removed and the RNA pellet was washed with 75% ethanol and air dried for 5 minutes. The final RNA product was suspended in 50µl RNase-free water. The concentration of the RNA samples was measured by a spectrophotometer at 260nm (Perkin Elmer, USA).

RNA was synthesized to cDNA in a 20µl reaction mixture containing, 5mM $MgCl_2$, 1mM of each dNTP, 2.5µM oligo $d(T)_{16}$ primers, 20 units of RNase

inhibitor (Applied Biosystems) and 50 units of MuLV reverse transcriptase (Applied Biosystems) at 42°C for 1 hour followed by 95°C for 10 minutes.

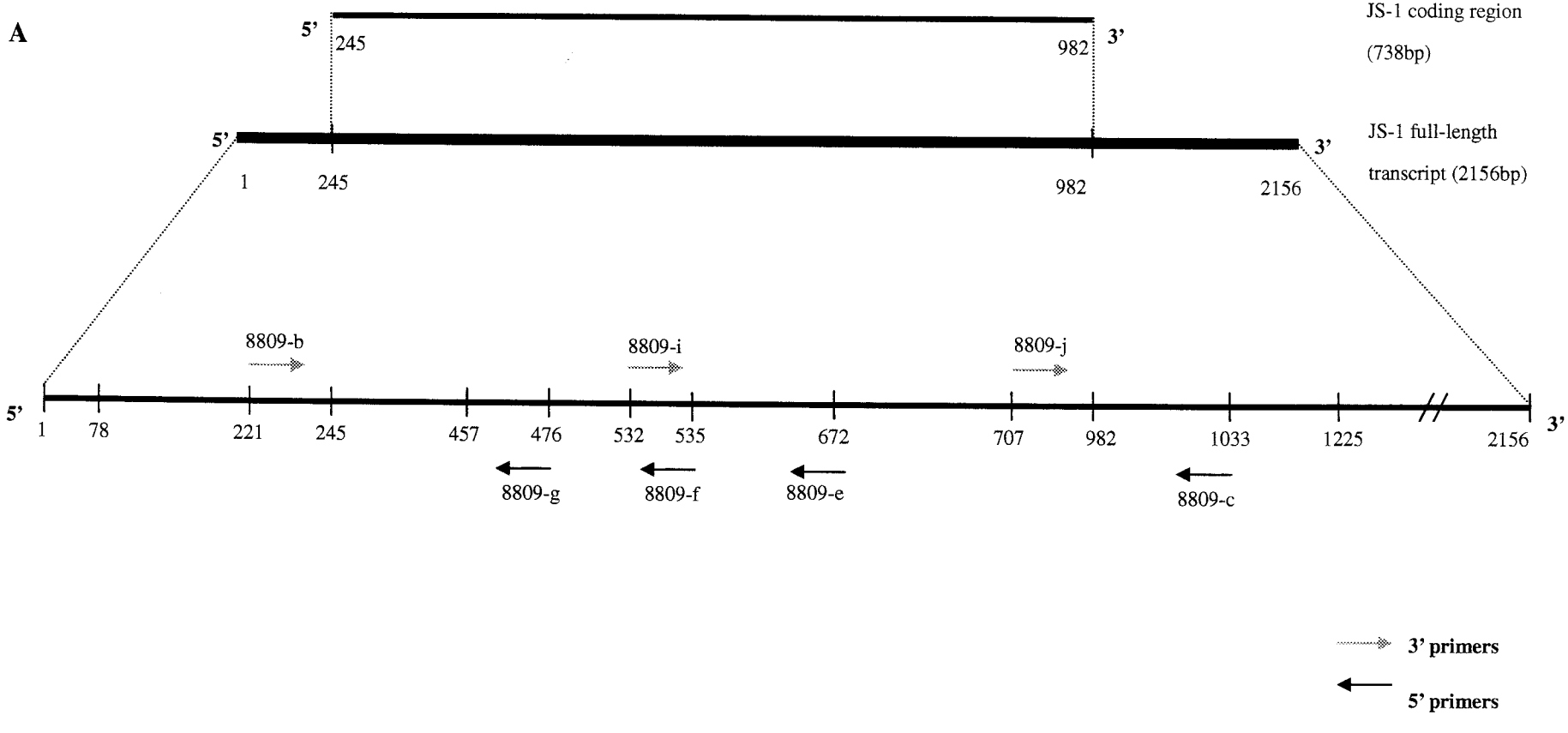
2.3 Characterization of JS-1 and JS-2 genes

2.3.1 3’ and 5’-rapid amplification of cDNA ends (RACE)

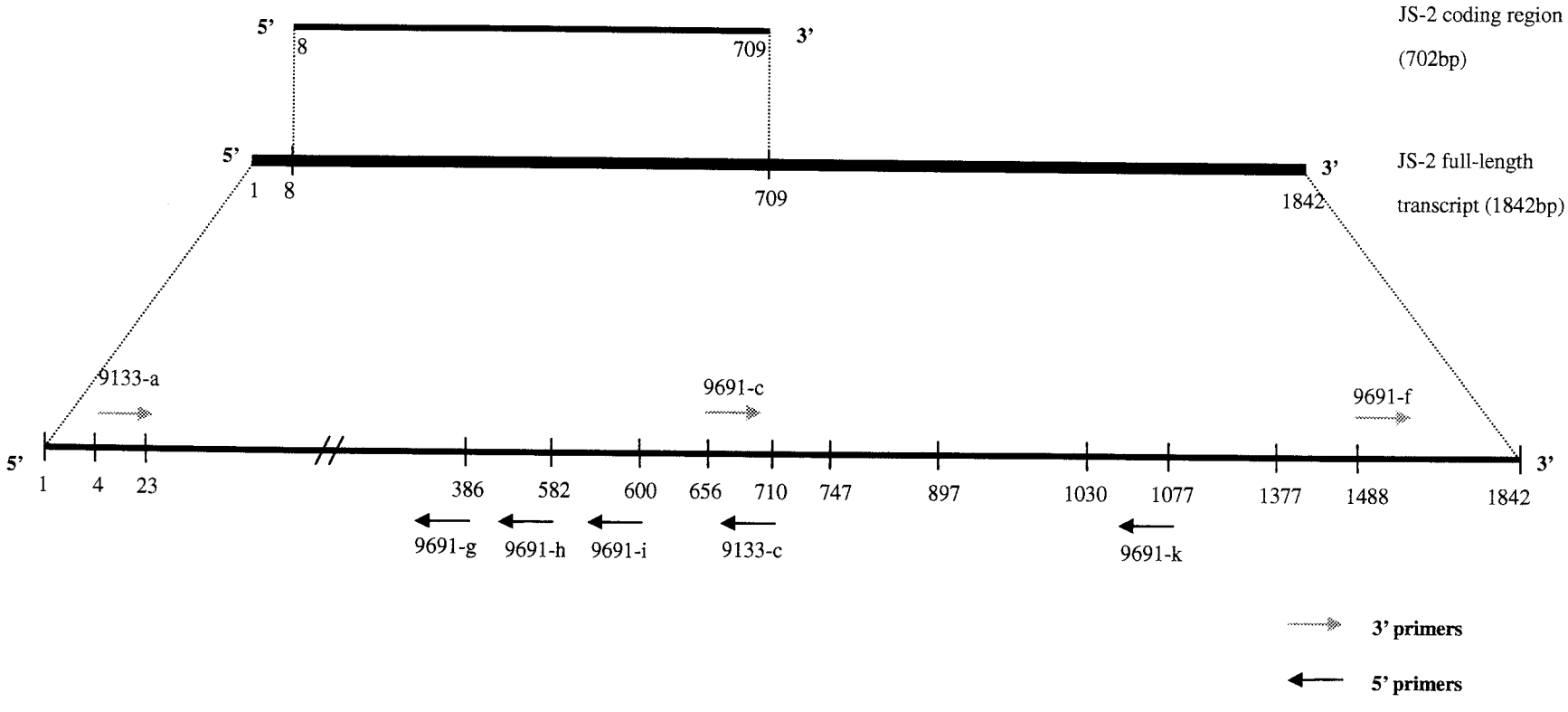
Total RNA sample of non-tumor esophageal epithelial cell line (NE3) was used for the RACE study to determine the full-length transcripts of JS-1 and JS-2 using the 5’/3’ RACE Kit (Roche). Figure 2A and Figure 2B show the positions of primers used in the present study of JS-1 and JS-2 respectively. Figure 2C shows the relative positions of JS-1 and JS-2 genes to known genes on chromosome 5p15.2. For the 3’RACE study, the first cDNA strands were synthesized from total RNA using olido dT anchor primer (5’ GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV 3’; V=A, C or G) and were purified using QIAEX II (Qiagen) gel extraction kit. The subsequent amplification of the purified cDNA was done using PCR anchor primer (5’ GAC CAC GCG TAT CGA TGT CGA C 3’) and 8809-i (5’ GTG GCT GAA AAC AAG AAA TG 3’) for the study of JS-1 or the 9691-f (5’ TCT TTG GTC CAT AAA TGG TG 3’) for the study of JS-2. For the 5’ RACE study, the first strand

cDNA was synthesized from the total RNA by using the primer 8809-e (5’ ATC ATC AAA TGA TGG ACA GC 3’) for JS-1 study or 9691-i (5’ AGC AAT AAC TCG TGC ATC AT 3’) for JS-2 study. The synthesized cDNA was then purified using the QIAEX II (Qiagen) gel extraction system. The purified cDNA was then tailed with dATP by terminal transferase and PCR amplification was done using the oligo dT anchor primer and 8809-f (5’ CCA CTC AGG GAA GAT AGA CC 3’) for JS-1 or 9691-h (5’ ATC CTC AAG TTC ACG TTC AA 3’) for JS-2. The nested PCR amplification of the first PCR was followed using PCR anchor primer and 8809-g (5’ AGT CTG GAA CAA TGG TTG TG 3’) for JS-1 or 9691-g (5’ ACT GCT GTG AAC CCT TTC TT) for JS-2. The PCR products were then electrophoresed in 2% agarose gel, containing 10ng/ml ethidium bromide, and visualized under UV.

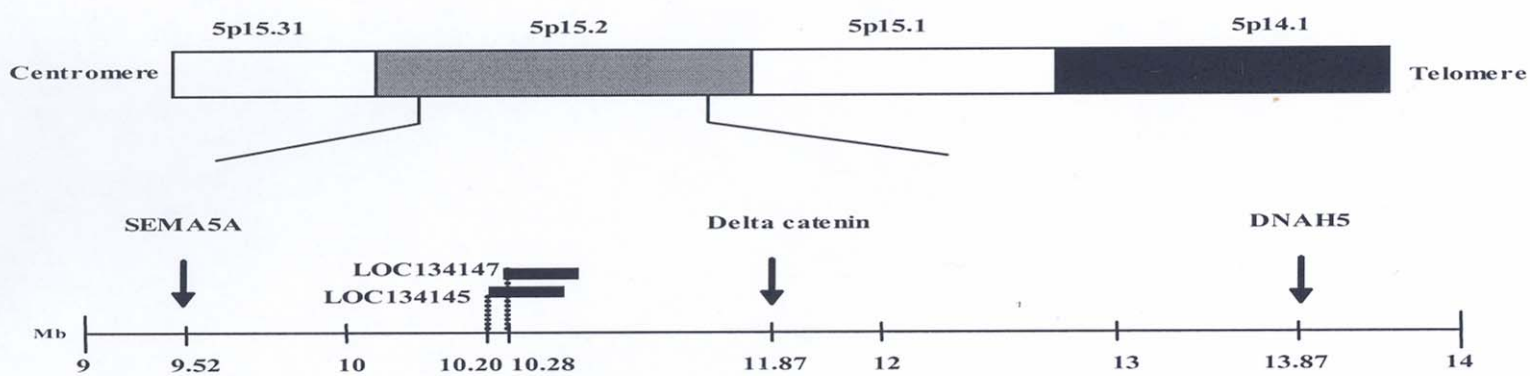
Figure 2. Schematic diagrams showing the positions of primers for **(A)** JS-1 and **(B)** JS-2 studies. The positions of primers are numbered according to the full-length cDNA sequences produced from 3'RACE and 5'RACE analysis. **(C)** The chromosomal positions of JS-1 and JS-2 genes relative to the known genes on chromosome 5p15.2. JS-1 and JS-2 are located at LOC134147 and LOC134145 respectively which are 5'upstream to delta catenin gene. The diagrams are not drawn to scale.


A
5'
245
982
3'
JS-1 coding region
(738bp)
5'
1
245
982
2156
3'
JS-1 full-length
transcript (2156bp)
8809-b
8809-i
8809-j
5'
1
78
221
245
457
476
532
535
672
707
982
1033
1225
2156
3'
8809-g
8809-f
8809-e
8809-c
3' primers
5' primers

B


5'
8
709
3'
JS-2 coding region
(702bp)
5'
1
8
709
1842
3'
JS-2 full-length
transcript (1842bp)
9133-a
9691-c
9691-f
5'
1
4
23
386
582
600
656
710
747
897
1030
1077
1377
1488
1842
3'
9691-g
9691-h
9691-i
9133-c
9691-k
3' primers
5' primers

C


5p15.31
5p15.2
5p15.1
5p14.1
Centromere
Telomere
SEMA5A
Delta catenin
DNAH5
LOC134147
LOC134145
Mb
9
9.52
10
10.20
10.28
11.87
12
13
13.87
14

2.3.2 Gel extraction

Target DNA bands from RACE were excised from the agarose gel with a clean, sharp scalpel and placed in a 1.5ml tube. 300µl of Buffer QXI (Qiagen) and 10µl of QIAEX II (Qiagen) were added to the sample. The sample was than incubated for 50°C for 10 minutes to solubilize the agarose and bind the DNA. The tube was vortexed every 2 minutes to keep QIAEX II (Qiagen) in suspension. The sample was then centrifuged for 30 seconds at 10,000g. The supernatant was removed and 500µl of Buffer QX1 (Qiagen) was added. The pellet was resuspended by vortexing and the sample was again centrifuged for 30 seconds at 10,000g. The supernatant was removed and the pellet was washed twice with 500µl Buffer PE (Qiagen). After removing the supernatant following the second wash, the pellet was air dried for 15 minutes. The pellet was then resuspended in 20µl water and incubated for 5 minutes. This was followed by centrifugation for 30 seconds at 10,000g. The supernatant was transferred into a clean tube and stored in -20°C.

2.3.3 PCR analysis on human ESCC specimens and their corresponding non-tumor tissues

Total RNA was extracted from ESCC cell lines, frozen ESCC tumor tissues and their corresponding non-tumor esophageal epithelial tissues using TRIzol (Invitrogen)/chloroform method and cDNA was synthesized as described in section 2.2. One seventh of the total RT-PCR mixture was amplified in a 20µl reaction containing 1µM of each primer, 1 unit of Taq polymerase (Promega), 0.2mM of each dNTP, 2mM $MgCl_2$ and the supplied PCR buffer (50mM Tris-HCL, 100mMNaCl, 0.1mM EDTA, 1mMDTT, 50% glycerol & 1% Triton X-100). PCR was performed at initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation for 1 minute, annealing for 1 minute at 50°C and 47°C for JS-1 and JS-2 genes respectively and extension at 72°C for 1 minute, followed by a final extension at 72°C for 6 minutes. The primers used to amplify the JS-1 and JS-2 transcripts to study the expression level of the two genes are as follows: For JS-1, the primers are 8809-j (5’ TAT GGC ATT GTC AAG GAT TC 3’) and 8809-c (5’ AGC AAA TTT TGG GAT GAA AG 3’). For JS-2, the primers are 9691-c (5’ ACA CAG TGT GGG CAT ATG AT 3’) and 9691-k (5’ TAT CGA GGC ATT AAG CCA TA 3’). Expression

of β-actin and glyceryl aldehyde-3 phosphate dehydrogenase (GAPDH) was used as internal controls. For β-actin the primers were, sense: 5'GTG GGC CGC TCT AGG CAC CAA 3' and antisense: 5'CTC TTT GAT GTC ACG CAC GAT TTC 3' (Clontech). For GAPDH the primers were GAPDH 1: 5' TGA AGG TCG GAG TCA ACG GAT TTG GT 3' and GAPDH 2: 5'CAT GTG GGC CAT GAG GTC CAC CAC 3' (Clontech). The PCR products were then electrophoresed in 1.5% agarose gel, containing 10ng/ml ethidium bromide, and visualized under UV. The intensity of PCR products was measured using Quantity One (Biorad) gel image analysis system. The signal for each transcript was standardized against that of the β-actin mRNA from each sample. JS-1 and JS-2 mRNA expression levels were calculated as [(JS-1 or JS-2/β-actin, tumor)] / [(JS-1 or JS-2/β-actin, paired non-tumor)] (Miyazono *et al.*, 2004; Cen *et al.*, 2004).

2.3.4 Gene expression study using multiple tissue cDNA (MTC) panel

Human MTC panels (Clontech) contain a set of normalized first-strand cDNA from different normal human tissues. PCR using a pair of gene specific primers allows analysis of gene expression analysis across many tissues. A

higher sensitivity of RT-PCR makes it possible to detect small amounts of transcripts which may not be able to be detected in Northern blots. It also gives a picture of gene expression variation between different tissues (Ruddy *et al.*, 1997). Expression of JS-1 and JS-2 was determined by RT-PCR study, as described in the previous section, on multiple tissue cDNA (MTC) panel (Clontech) of the human digestive tract that includes 10 separate gastrointestinal organs comprising the whole esophagus, stomach, duodenum, ileocecum, jejunum, colon ascending, colon transverse, colon descending, rectum and liver. For every PCR reaction, cDNA from each sample of the panel was amplified at initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation for 1 minute, annealing for 1 minute at 50°C and 47°C for JS-1 and JS-2 genes respectively and extension at 72°C for 1 minute, followed by a final extension at 72°C for 6 minutes. β-actin was used to normalize the cDNA amount in each reaction. The PCR products were electrophoresed in 1.5% agarose gel, containing 10ng/ml ethidium bromide, and visualized under UV.

2.3.5 Cell transfection studies and transformation assay

2.3.5.1 Preparation of constructs for expression study

With reference to the results of the RACE study, the full-length cDNA sequences of JS-1 and JS-2 were worked out (Figures 6A and 6B respectively). The coding sequences of the two genes were produced from the cDNA of NE3 cell line by PCR using primers 8809-b (5' ACA AGC CCG ACT TAA ATC TC 3') and 8809-c (5' AGC AAA TTT TGG GAT GAA AG 3') for JS-1 or primers 9133-a (5' CGC GAT GGA GGG AGG AG 3') and 9133-c (5' AAG TTT ATG CTT GAA TGG GCA GCT 3') for JS-2 (Figures 2A and 2B respectively). The PCR products were purified using QIAEX II (Qiagen) gel extraction system. DNA sequencing from both directions was done on the coding regions to confirm their sequence identities. JS-1 coding region was cloned into pGEM-T Easy vector (Promega) (Figure 3) by NotI (Promega) restriction enzyme digestion and then subcloned into pcDNA3.1(+) vector (Invitrogen) (Figure 4) but sequencing results from both directions showed reverse orientation of the JS-1 clone. The JS-1 clone was then subcloned into pcDNA3.1(-) vector (Invitrogen) by BamHI (Promega) and ApaI (Promega)

restriction enzyme digestion and sequencing from both directions confirmed correct orientation of the clone. JS-2 coding region was cloned into pGEM-T Easy vector by EcoRI (Promega) restriction enzyme digestion and then subcloned into pcDNA3.1(+) vector. Sequencing from both directions confirmed correct orientation of the clone.

The following sections (2.3.5.1.1 – 2.3.5.1.4) will describe in detail the steps of the cDNA cloning. Briefly, coding regions of JS-1 and JS-2 and the vectors in which they were cloned into were digested by their respective restriction enzymes. The restriction enzyme digested products (cDNA and vector) were electrophoresed in 1.5% gel and target bands were excised from agarose gel and purified by QIAEX II (Qiagen) gel extraction system. The purified restriction enzyme digested cDNAs and vectors were then ligated and transformed into DH5α (Clontech) competent cells followed by isolation of positive colonies. Plasmid purification and sequence verification confirmed the single insert with appropriate direction for expression.

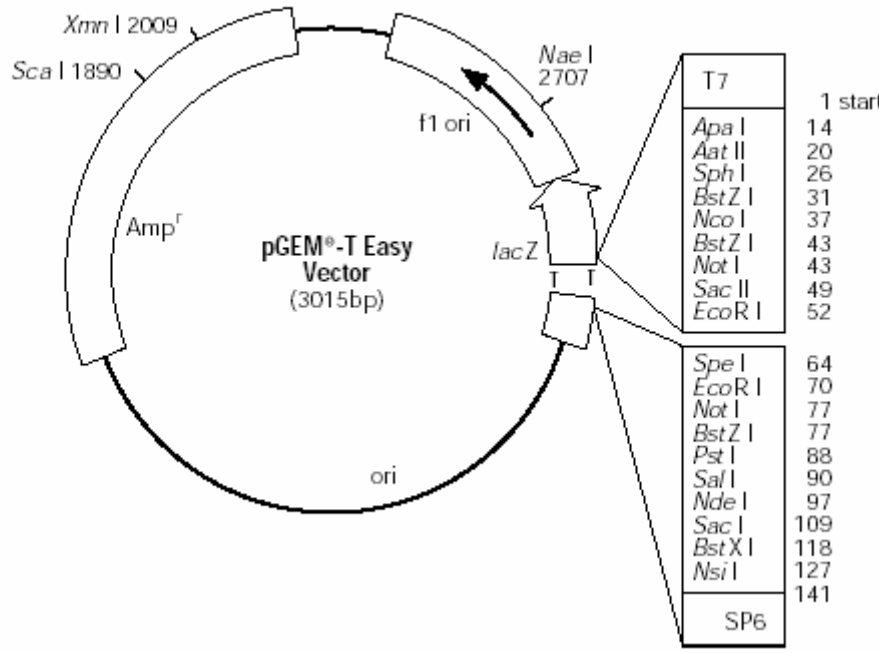


Figure 3. Schematic map of pGEM-T Easy vector showing the positions of its various elements. The multiple cloning site is on the right. f1 origin: for rescue of single stranded DNA; lac Z: encodes β-galactosidase and allows insertion screening by inactivation of lacZ gene; Ori: origin of replication to allow the plasmid to multiply within and semi-independently of its host; Amp^r: Ampicillin resistant gene for selection of the plasmid in *E. coli.* Adopted from Promega <http://www.promega.com>

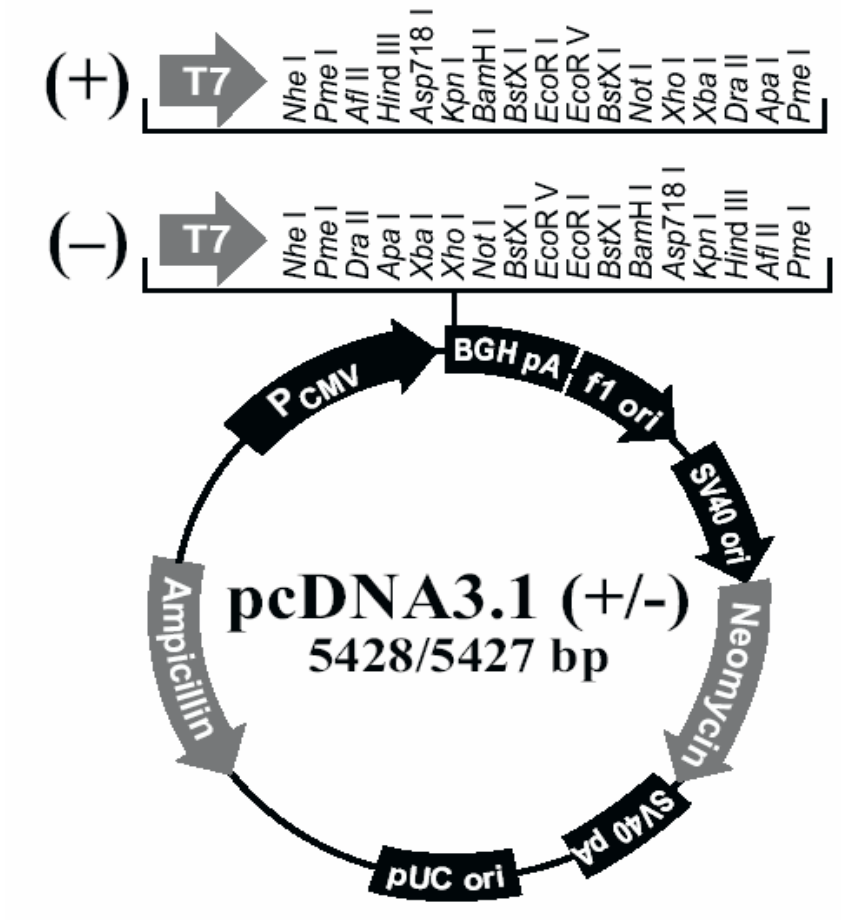


Figure 4. Schematic maps of pcDNA3.1(+) and pcDNA3.1(-) vectors showing the positions of different features for their functions. The multiple cloning sites are shown at the top. PCMV: human cytomegalovirus promoter for ensuring high level of expression of insert sequence; BGH pA: bovine growth hormone polyadenylation signal; f1 origin: for rescue of single stranded DNA; SV40 ori: SV40 promoter and origin allow efficient expression of neomycin resistance gene; Neomycin: neomycin (G418) resistance gene for growth selection; SV40 pA: Allows transcription termination and polyadenylation signal for the neomycin gene; pUC ori: Allows for high copy-number changes in E. *coli*; Ampicillin: Ampicillin resistant gene for selection of the plasmid in *E. coli.* Adopted from Invitrogen <http://www.invitrogen.com>

2.3.5.1.1 Restriction enzyme digestion

A 15µl reaction containing 2µl of 10X supplied restriction enzyme buffer (Promega), 5µl cDNA/plasmid vector, 1 unit of restriction enzyme and 7µl water was incubated for 3 hours at 37°C. The reaction was electrophoresed and the linearised DNA was purified from agarose gel by QIAEX II (Qiagen) gel extraction system.

2.3.5.1.2 Ligation

Following purification of the linearised DNA, a 20µl reaction of 1µl ligase (Promega), 2µl 10X ligase buffer (Promega), 16.5µl linearised cDNA and 0.5µl plasmid was incubated at 4°C for 16 hours.

2.3.5.1.3 Transformation

Following ligation, the 20µl ligation reaction was added to 200µl of DH5α (Clontech) competent cells. The cells were put on ice for 20 minutes. They were then heat shocked in 42°C water bath for 90 seconds and put on ice for 2 minutes. 800µl of LB broth was added and the reaction was incubated for three hours at 37°C. 200mg/ml IPTG and 40µg/ml of Xgal were later added. 340µl of the reaction was plated onto an agar plate containing 100µg/ml ampicillin. The plate was incubated

at 37°C for 16 hours. Following transformation, individual colonies were picked and incubated in 5ml LB broth containing 100µg/ml ampicillin and incubated at 37°C for 16 hours.

2.3.5.1.4 Plasmid extraction

Following transformation, 2ml of overnight culture was transferred into a 2ml tube and centrifuged at 12,000g for 1 minute. The supernatant was discharged and 200µl of cell resuspension solution (Biorad) was added, the tube was then vortexed to resuspend the pellet. 250µl of cell lysis solution (Biorad) was added and the solution was mixed by inverting the tube 10 times. 250µl of neutralization solution (Biorad) was then added and the tube was inverted 10 times again and centrifuged at 12,000g for 5 minutes. 200µl of matrix (Biorad) was dispensed into the supernatant and the solution was pipetted up and down twice. All the solution was then transferred into a 1.5ml tube with a spin column. This was followed by centrifugation at 12,000g for 1 minute. The supernatant was discharged and the pellet was washed twice with 500µl wash buffer (Biorad) and centrifuged at 12,000g for 1 minute. The supernatant was discharged and the spin column was placed in a new 1.5ml collection tube. 120µl of deionized water was added and centrifuged at

12,000g for 1 minute. The spin column was discarded and the eluted DNA was stored in -20°C.

2.3.5.2 Transfection of NIH 3T3 cells

NIH 3T3 cells were cultured in DMEM medium supplemented with 10% FBS. Subconfluent NIH 3T3 cells ($3x10^5$ cells seeded in triplicate of 6-well plate) were transfected with 1μg of the following plasmids: (a) an empty vector of pcDNA3.1(-) as a negative control; (b) pcDNA3.1-JS-1 or pcDNA3.1-JS-2 constructs; (c) pcDNA3.1-H-*ras*V12 construct as a positive control, which was kindly provided by Dr. Cao L of the Department of Microbiology, The University of Hong Kong. NIH 3T3 cells were transfected using a mixture composed of 1μg plasmid : 6μl FuGENE 6 (Roche Diagnostics) transfection reagent. Seventy-two hours after transfection, cells were re-plated and selected with 300μg/ml G418 antibiotic (JRH Biosciences) for 2 weeks. JS-1 and JS-2 transfected cells were then harvested by trypsinization to confirm expression of JS-1 and JS-2 genes by RT-PCR as described in the previous section using β-actin expression as control.

2.3.5.3 Cell growth assay

$1x10^5$ parental NIH 3T3 cells and NIH 3T3 cells transfected with (a) pcDNA31.(-) vector only (named as 3T3/vec), (b) JS-1 (named as 3T3/JS-1) and (c) pcDNA3.1-H-rasV12 (named as 3T3/ras) were grown in $75cm^2$ culture flasks with DMEM medium supplemented with 10% FBS and with or without the addition of 300µg/ml G418 antibiotic. Cells were trypsinised and counted using a haemocytometer (Boeco, Germany) on indicated days by the dye-exclusion method. When the cells were in a log growth phase, the population doubling time (dt) was determined by the formula $dt = lg2/lg(Ct/C_0)\times t$, where t is the time between cell counts Ct and C_0, C_0 is the initial count, and Ct is the count after time t (Ma *et al.*, 2004).

2.3.5.4 Soft agar assay

For the soft-agar assay, the procedures were followed as previously described (Cowley *et al.*, 1994). Each well (36mm) of a 6 well plate was coated with 2ml soft agar as the bottom layer. The bottom layer was a mixture of 20% 2xDMEM, 50% DMEM, 10% FBS, and 20% 2.5% agar. This mixture was combined at 45°C. $2x10^4$ parental NIH 3T3 cells and transfected cells were suspended in 3ml 1xDMEM and mixed with 6ml of agar mixture as the top layer. The transfected cells in soft

agar were incubated at 37°C for 16 days. The colony formation in each preparation with the clones having more than 20 cells was observed under microscope.

2.3.5.5 Foci formation assay

For the foci formation assay, $1x10^4$ of parental NIH 3T3 cells, 3T3/vec cells, 3T3/JS-1 cells and 3T3/ras cells were seeded in 6-well plates and grown to confluence. The cells were maintained in DMEM medium supplemented with 10% FBS, with or without 300μg/ml G418 antibiotic. The medium was replaced with fresh medium every 3 days. The cells were stained with methylene blue after 1 day, 3 days and 14 days for observation.

2.3.5.6 Tumorigenicity test

Approximately $1x10^6$ 3T3/JS-1 cells were subcutaneously injected into each flank of five female athymic nude mice after selection in 300μg/ml G418 antibiotic for 14 days. Parental NIH 3T3 cells, 3T3/vec cells and 3T3/ras cells were also subcutaneously injected into athymic nude mice and served as controls. Formation of subcutaneous tumor was monitored for 30 days. The tumors were dissected and fixed in formalin for histopathological examination. Total RNAs and cDNAs of the

parental NIH 3T3 cells, 3T3/vec cells, 3T3/JS-1 cells and the subcutaneous tumors collected were prepared, and the level of JS-1 expression was examined by RT-PCR as described in the previous sections using GAPDH expression as the control.

2.4 Bioinformatics analysis on JS-1 and JS-2 transcripts and proteins

Nucleic acids and amino acids homology searches were performed by using Basic Local Alignment Search Tool (BLAST) software in National Centre of Biotechnology Information (NCBI). ScanProsite (http://au.expasy.org) was used to scan the protein sequence of JS-1 and JS-2 for the occurrence of patterns available in the PROSITE database.

CHAPTER 3 RAPID AMPLIFICATION OF cDNA ENDS (RACE) AND EXPRESSION STUDIES ON JS-1 AND JS-2

3.1 Selection of samples and controls

A non-tumor esophageal epithelial cell line, NE3, and eleven ESCC cell lines including SLMT-1 (Tang *et al.*, 2001a), HKESC-3 (Hu *et al.*, 2002), and KYSE 30, 70, 140, 150, 180, 410, 450, 510, 520 (Shimada *et al.*, 1992) were used to study for JS-1 and JS-2 expression by RT-PCR. NE3, served as a control. For RT-PCR study on primary ESCC for expression of JS-1 and JS-2 genes, 27 ESCC specimens (patient nos.1-27) along with their corresponding non-tumor tissues, were used for JS-1 study and 22 ESCC specimens (patient nos. 6-27) along with their corresponding non-tumor tissues, were used for JS-2 study due to insufficient ESCC tissue availability. The clinical characteristics of ESCC specimens studied are listed in Table 5.

3.2 Results

3.2.1 3’ and 5’ RACE

Figure 5 shows the products of 3’ and 5’ RACE analysis. The 3’ and 5’ RACE analysis was performed on mRNA of NE3 cell line. For JS-1, PCR bands (products a and b) could be generated from the second round of PCR of each 3’ and 5’ RACE

reactions respectively. PCR products c and d were produced from the 3' and 5' RACE reactions of JS-2. The RACE products of JS-1 and JS-2 were then directly sequenced to determine the 3' and 5' cDNA sequences of the 2 genes. Figure 6A and Figure 6B show the full-length sequences of JS-1 and JS-2 cDNA respectively based on the RACE analysis together with the predicted amino acid sequence and the 3' and 5' non-coding sequences. The first ATG codon for JS-1 and JS-2 are at positions 245 and 8 respectively. They are associated with a Kozak sequence and are therefore deduced to be the start codons. The sequence that surrounds the start codon in JS-1 and JS-2 has a consensus sequence for initiation of translation by eukaryotic ribosomes with a purine at position -3 and a guanine following the ATG codon (Kozak M, 1987; Kozak M, 1990; Kozak M, 1991). The JS-1 transcript is composed of 2156 nucleotides with 738bp of coding sequence and 1174bp of 3' and 244bp of 5' non-coding sequences. According to the PolyA signal predictor program available from the Institute for Biomedical Technologies, National Research Council (http://125.itba.mi.cnr.it/~webgene/wwwHC_polya.html), the polyA signal for JS-1, AATAAA (AAUAAA), is at position 2132-2137. JS-1 is also predicted to encode a protein of 245 amino acids. The JS-2 transcript is composed of 1842 nucleotides with 702bp of coding sequence and 1133bp of 3' and 7bp of 5' non-coding sequences. The polyA signal, ACTAAA (ACUAAA), for JS-2 is at position 1799-1804 and JS-2 is predicted to encode a protein of 233 amino acids.

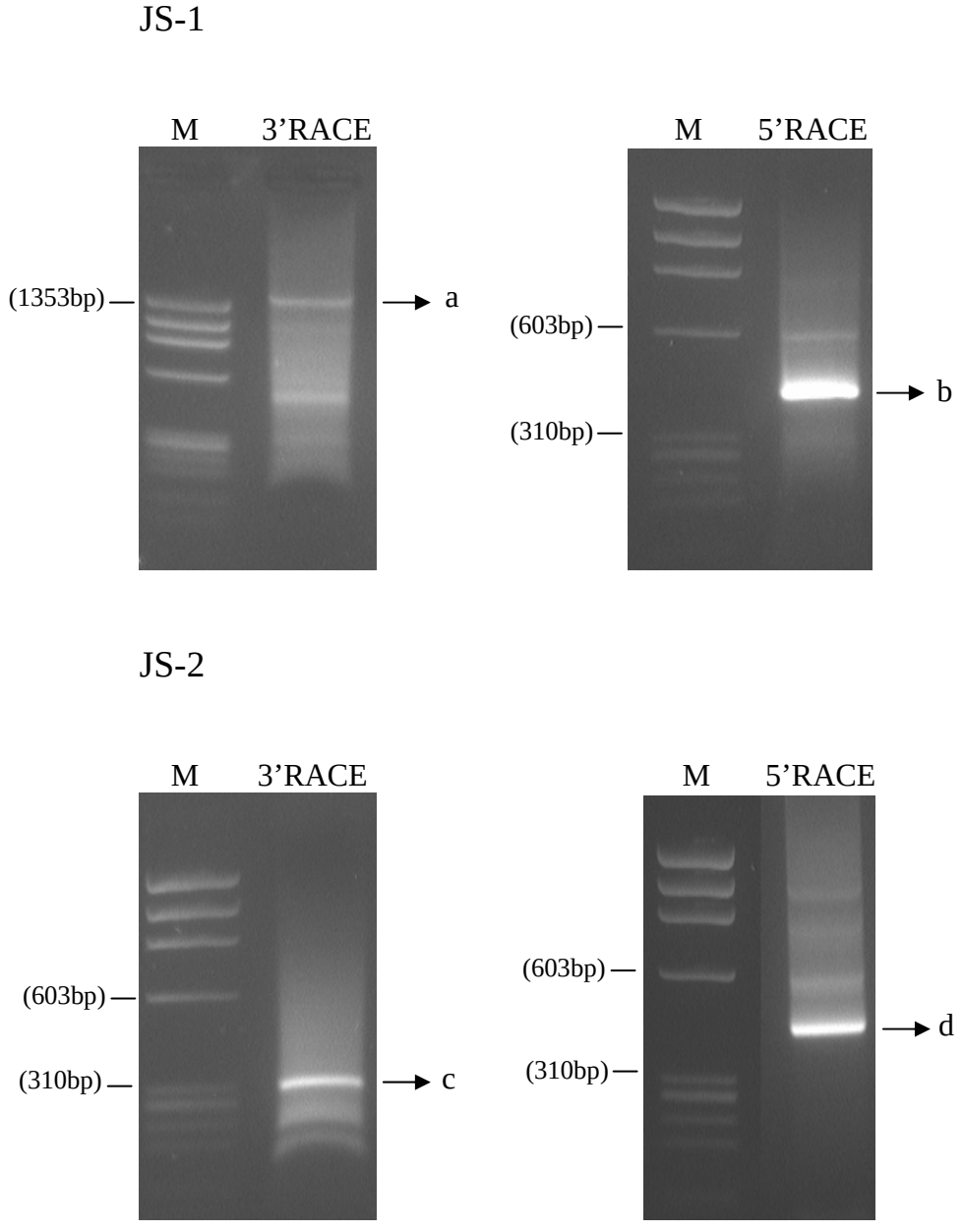


Figure 5. 3' and 5' RACE analysis for JS-1 and JS-2. For JS-1, bands a and b were generated by the 3'RACE and 5'RACE reactions respectively. For JS-2, bands c and d were produced as the 3'RACE and 5'RACE products. M: molecular size marker (ϕ174/Hae III cut).

```
                                                                    g
2     actttgcagatttctcttcccccaggcctccctcctccacctctccgccccctccgggct
62    tggctctcccaggaggctacgactggagccactggtcccgcaggatccccgcgtcctcgg
122   tcgccgcgtccacgtccctctcgcgtccccgcccggcgccacgccgcctccctctgggtt
182   cggcctccgcgcggtgcagcgcagttctcaggccgcgggacaagcccgacttaaatctct
242   gca atg gctaacgaagcttatccttgtccgtgtgacattggccacagacttgagtatgga
1        M  A  N  E  A  Y  P  C  P  C  D  I  G  H  R  L  E  Y  G
302   gggctaggccgtgaagttcaagtcgagcacatcaaggcttatgtcaccaaatcccccgtt
20    G  L  G  R  E  V  Q  V  E  H  I  K  A  Y  V  T  K  S  P  V
362   gatgcaggcaaagctgtgattgtcattcaagatatatttggctggcagttgcccaatacc
40    D  A  G  K  A  V  I  V  I  Q  D  I  F  G  W  Q  L  P  N  T
422   agatatatagctgacatgatctcaggaaatggatacacaaccattgttccagacttcttt
60    R  Y  I  A  D  M  I  S  G  N  G  Y  T  T  I  V  P  D  F  F
482   gtagggcaagagccttgggacccctctggcgactggtctatcttccctgagtggctgaaa
80    V  G  Q  E  P  W  D  P  S  G  D  W  S  I  F  P  E  W  L  K
542   acaagaaatgcccagaagatcgatagagagatcagtgctatcttgaagtatctgaaacaa
100   T  R  N  A  Q  K  I  D  R  E  I  S  A  I  L  K  Y  L  K  Q
602   cagtgtcatgcccagaaaattggcatcgtgggattctgctggggtggaactgctgtccat
120   Q  C  H  A  Q  K  I  G  I  V  G  F  C  W  G  G  T  A  V  H
662   catttgatgatgaaatactcagaattcagggcaggggtgtccgtctatggcattgtcaag
140   H  L  M  M  K  Y  S  E  F  R  A  G  V  S  V  Y  G  I  V  K
722   gattctgaagacatttacaatttaaagaaccccactttgttcatttttgctgaaaatgat
160   D  S  E  D  I  Y  N  L  K  N  P  T  L  F  I  F  A  E  N  D
782   gttgtgattccactcaaggacgtatctttgctgactcagaagttgaaagaacactgcaaa
180   V  V  I  P  L  K  D  V  S  L  L  T  Q  K  L  K  E  H  C  K
842   gttgaatatcaaattaaaacattttctgggcagactcatgggttcgtgcatcggaagaga
200   V  E  Y  Q  I  K  T  F  S  G  Q  T  H  G  F  V  H  R  K  R
902   gaagattgctcacctgcagacaagccctacattgacgaggccagaaggaatttaattgag
220   E  D  C  S  P  A  D  K  P  Y  I  D  E  A  R  R  N  L  I  E
962   tggctgaacaagtacatg tag caagaatcaagggcaagccttcctagaatagctttcatc
240   W  L  N  K  Y  M
1022  ccaaaatttgcttggaaatagttagatcatttgatttaattttcacttttataaaataag
1082  tgtaggaatcctaaaattgattatttcatttgaaacacaaattcagtaggacgtaatgca
1142  tgaaataatttaatttttgacatgtacatcgaatcataatttaaaaacaaggtctgacca
1202  ggtgtagtgcctcatgcctgtaattccagcactttgggaggccaaagtgggtggatcacc
1262  tgaggtcaggagtttgagaccagcctggccaacatggtgagaccccatctctacaaaaaa
1322  tacaaaaattagcctggtgtggtggtgcacacctgtagtcccagctacttgggaggctga
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1442  tactgtactctagcctgggcagcagagtgagaccctgtctcaaaaataaataagtaaata
1502  aaataaataaataaaataaaaaccaggtcagtacctggagaatttgaatgatagagaatg
1562  tagagtaataccctaattattagttaaaacctacaggccgggtgyggtggctcacgcctg
1622  taatcccagcactttgggaggccgaggtgggtggatcacttgaggtcaggagttcaagac
1682  cagcctggccaacatggtgaaaccccatctctactaaaaatataaaaatcagccgggcat
1742  ggtggcatgtgcctgtaatcccagctactcaggagtctgaggaggagaatcacttgaacc
1802  tggaggcagaggttgcagtgagtcgaggttgcgctactgcactccagcctggacaacaga
1862  gggagactctgtctcaaaaaaaaaaaacctacagctgttcaaggaccagctgacaggtca
1922  agtgtggccttttctggtctttgaacacatcatagaaagtgacaaatgctgcaaagccat
1982  gaagaacatgaactataaacgggtagactaactgcccagcttagacacttatctatgcca
2042  caaaacagctgaatttgtcacatttatatattgcaatatgggaagtattgagatcaaaac
2102  aggattccattgacctaattatattaagct aataaa ctaatttttgaatttttg
```

Figure 6A. Nucleotide and predicted amino acid sequence of JS-1 based on 3' and 5'RACE analysis. The translation initiation codon (ATG) is underlined and italicized, the stop codon (TAG) is underlined and the PolyA signal (AATAAA) is in bold.

```
                                                                  ctcc
5     gcgatggagggaggaggaggtatacccctagaaacacttaaagaagaaagtcagtcaaga
1        M  E  G  G  G  G  I  P  L  E  T  L  K  E  E  S  Q  S  R
65    catgttctacctgcaagttttgaagtcaacagtttgcagaaaagcaactgggggttctta
0     H  V  L  P  A  S  F  E  V  N  S  L  Q  K  S  N  W  G  F  L
125   cttactgggcttgtgggtggcaccctggtggctgtgtacgctgtagccacgccgtttgta
40    L  T  G  L  V  G  G  T  L  V  A  V  Y  A  V  A  T  P  F  V
185   acgccagcccttcgaaaagtctgtttgccatttgtacctgcaactatgaagcagattgaa
60    T  P  A  L  R  K  V  C  L  P  F  V  P  A  T  M  K  Q  I  E
245   aatgttgtgaaaatgttgcgatgccgaagaggatcccttgtggacatcggtagtggggac
80    N  V  V  K  M  L  R  C  R  R  G  S  L  V  D  I  G  S  G  D
305   ggacgcattgtcatagcggctgcgaagaaagggttcacagcagttggttatgaattaaac
100   G  R  I  V  I  A  A  A  K  K  G  F  T  A  V  G  Y  E  L  N
365   ccatggctagtttggtattccagataccgcgcttggcgagaaggtgtgcatggatctgcc
120   P  W  L  V  W  Y  S  R  Y  R  A  W  R  E  G  V  H  G  S  A
425   aaattttatatttcagatttgtggaaggttacttttcgcagtactcgaacgttgttatt
140   K  F  Y  I  S  D  L  W  K  V  T  F  S  Q  Y  S  N  V  V  I
485   ttcggtgtgcctcagatgatgctgcagttggagaagaaacttgaacgtgaacttgaggat
160   F  G  V  P  Q  M  M  L  Q  L  E  K  K  L  E  R  E  L  E  D
545   gatgcacgagttattgcttgccggttccctttcccacattggactccagaccacgtcacg
180   D  A  R  V  I  A  C  R  F  P  F  P  H  W  T  P  D  H  V  T
605   ggggaggggatagacacagtgtgggcatatgatgcaagcactttagaggccgtgaaaag
200   G  E  G  I  D  T  V  W  A  Y  D  A  S  T  F  R  G  R  E  K
665   aggccctgtacatcgatgcatttccagctgcccattcaagcataaactttactgggagtg
220   R  P  C  T  S  M  H  F  Q  L  P  I  Q  A
725   tttttctgaaatattgtagtcttccctgatcttgtagagacttacagctgtgtccttagc
785   aaaggagcataattgtctttggtttggaatgagaaattactattactttccttaactgtt
845   ggagaaaacaaacgaagaaaaacaggtagatttaaaatctgcttttgatgctattttctt
905   aaaaattgtacttactgcctttccatgcacctggatgcatgaaaagatgaataagtgatg
965   gtcatggttatgggtaggaaaataagatgccaatagctcttcacattataaacaatatgg
1025  cttaatgcctcgataaggagaaatgatttttaaagacagtatatattaccaactgtttgt
1085  agtatcctataactttgttcatacttaagttgtatctctttccttttgtacttcatggc
1145  aaaattaattttctaaaggattcagagaggcgtttatattttataacatttattagaaat
1205  aatttgttccaccactagattactgtattttatggaatctaagctgtatatgtgtacatt
1265  tgtatacatgtatgtgtaaatgtaagcctgacgtgggttcagattccagttttgtcatca
1325  gcaagaggcctcggacccatgacttactgcctctgagccttggctttctgtgtatctctg
1385  ccactgggataatggtacacataacagtggagtggggaccaatttctaaaactcatggca
1445  cctagtctttggtccataaatggtgtttgttattttagacactgagaaaaatcttattca
1505  cagaagttgcagattatttctgctagtcacttttttatttcttgagagctgccactcatt
1565  taatatttctcatttatgagaagagacttgactgatttagataaaagacgaattaaggaa
1625  aacctttgaacgtgagtagacaagcttcatatatctgtaaagtgaatattgaatggtagc
1685  ttgagtcattcgtacttatcacagaaaagaattgaactcccagtcatacacctgtaactg
1745  aaataccaaactgtcttaatggagatgtaatgaagtaagatggtcacatcttta**actaaa**
1805  cttataaaaattgaaaaaaaaaaaaaaaaaaaaaaaa
```

Figure 6B. Nucleotide and predicted amino acid sequence of JS-2 based on 3' and 5'RACE analysis. The translation initiation codon (ATG) is underlined and italicized, the stop codon (TAA) is underlined and the PolyA signal (ACTAAA) is in bold.

3.2.2 Semi-quantitative PCR protocol saturation determination

To ensure that the semi-quantitative RT-PCR protocol was not saturated, a standard curve was drawn based on the following PCR reactions: a) multiplex PCR of JS-1/JS-2 and β-actin; b) β-actin PCR only; c) JS-1/JS-2 PCR only. In all the semi-quantitative RT-PCR experiments, 3.4µg/µl of RNA was used. As shown in Figure 7, all RT-PCR protocols reached saturation at concentrations greater than 3.4µg/µl. Thus, the semi-quantitative RT-PCR protocol used for this study was not saturated and it was sensitive to change in conditions.

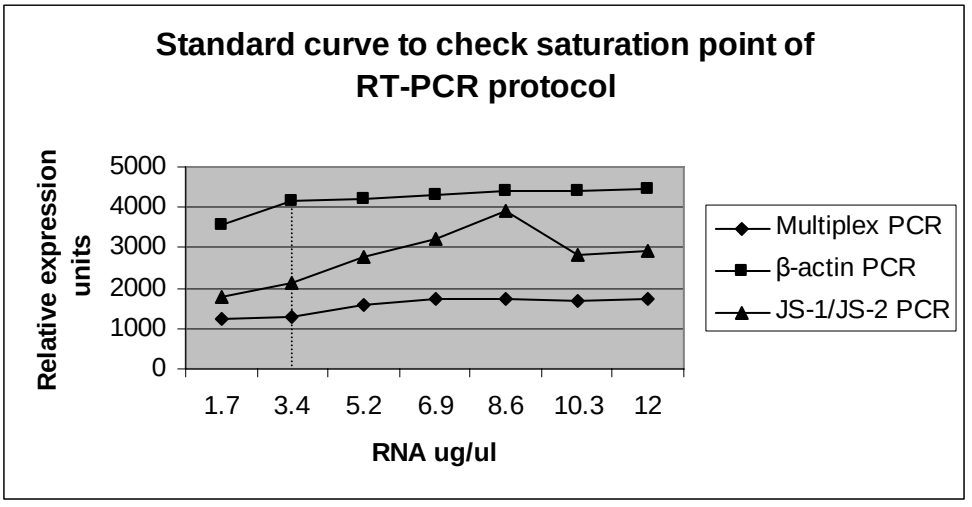


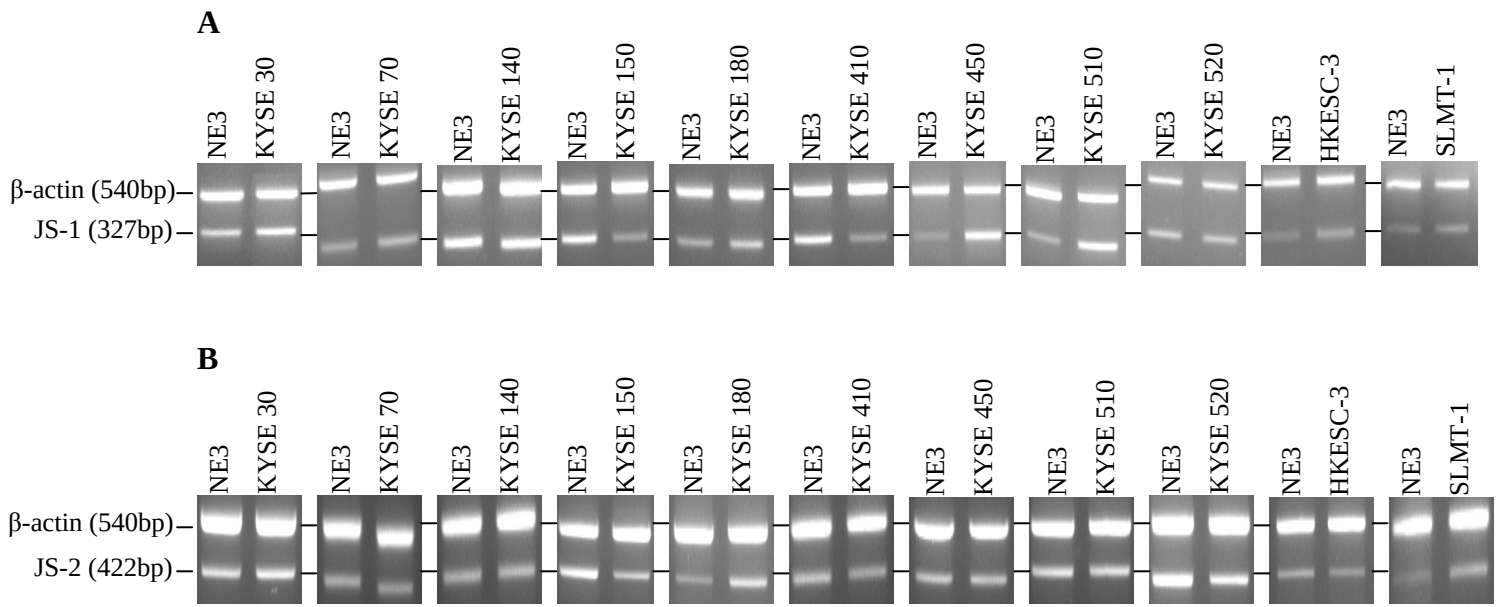
Figure 7. Standard curve to determine semi-quantitative RT-PCR protocol saturation in a) multiplex PCR of JS-1/JS-2 and β-actin; b) β-actin PCR only; c) JS-1/JS-2 PCR only. PCR saturation occurred at RNA concentrations greater than the working RNA concentration i.e. 3.4µg/µl.

3.2.3 JS-1 and JS-2 expression in human ESCC cell lines, tissue specimens and corresponding non-tumor tissues

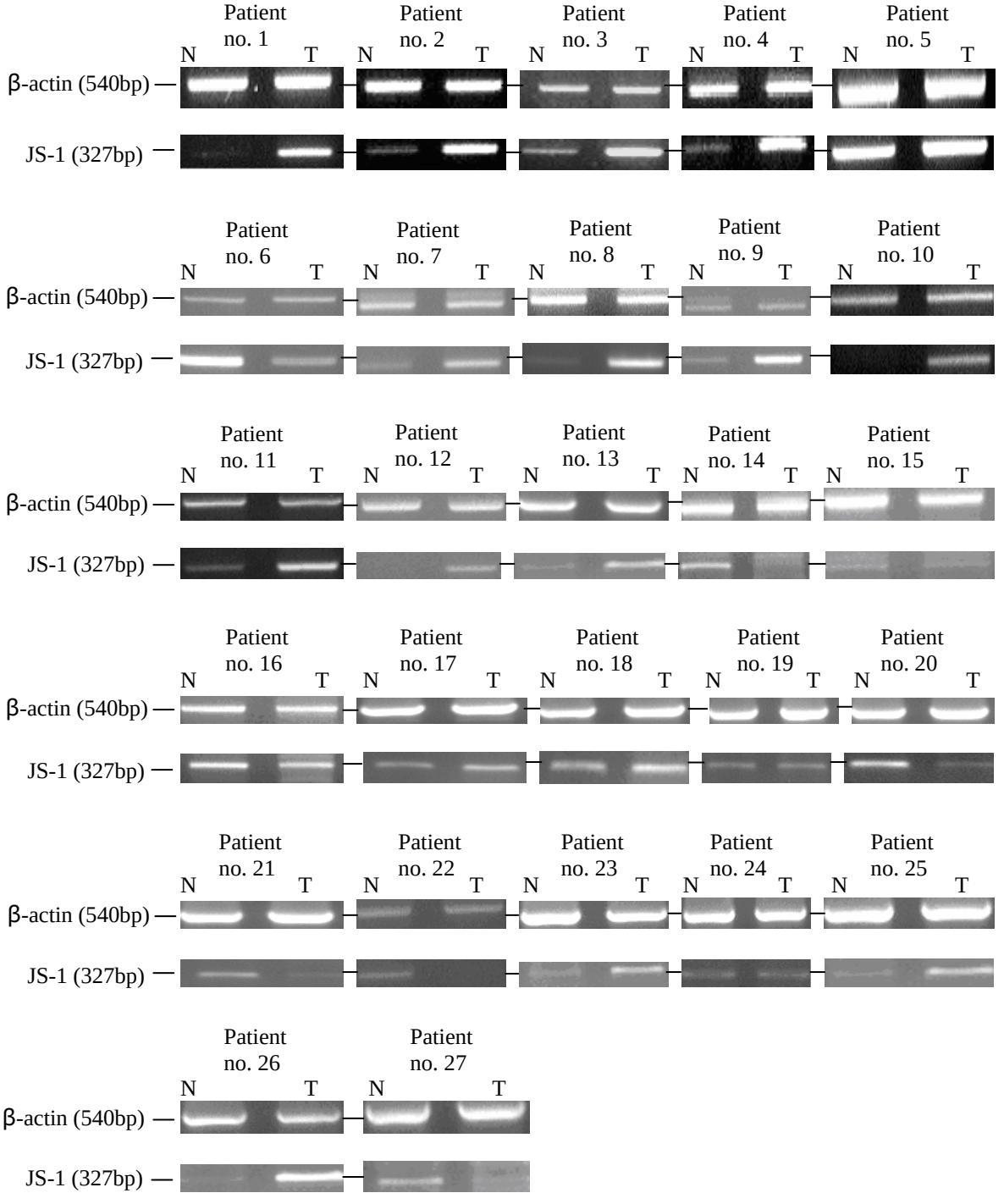
Expression of JS-1 and JS-2 novel genes was examined by semi-quantitative RT-PCR. The ratio of expression level of JS-1 and JS-2 in ESCC cell lines (Table 3) and tissue specimens (Table 4) was calculated as described in Materials and Methods, section 2.3.3. RT-minus RNA did not generate any target bands using the same primers for JS-1 and JS-2 study under the same PCR conditions. The expression was regarded as upregulation when the [(JS-1 or JS-2/β-actin, tumor)] / [(JS-1 or JS-2/β-actin, paired non-tumor)] ratio was > 1.2; between 0.8 to 1.2 was regarded as no change; and < 0.8 was regarded as down regulation (Zhou *et al.*, 2005). Figure 8A shows 45% (5/11; KYSE 30, 450, 510, HKESC-3 and SLMT-1) of ESCC cell lines overexpressing JS-1 gene and according to Figure 8B, only 18% (2/11; KYSE 180 and SLMT-1) of ESCC cell lines overexpressed JS-2 gene. Figures 8C and 8D show JS-1 and JS-2 overexpression in ESCC specimens respectively. As a whole, 56% (15/27) of ESCC specimens overexpressed JS-1 gene and only three out of 22 (14%) specimens showed overexpression of JS-2 gene. Twenty seven ESCC specimens were studied for JS-1 expression and only 22 specimens were studied for JS-2 expression because of insufficient ESCC tissue availability. The histopathological features and the expression patterns of JS-1 and JS-2 in ESCC specimens studied are summarized in Table 5. According to Figure 8E, JS-1 was expressed in all the normal tissues of the gastrointestinal system with

esophagus expressing relatively lower level than other gastrointestinal organs studied. JS-2 was expressed only in the stomach, ileocecum, jejunum and expression level was relatively lower in the rectum (Figure 8E).

Figure 8. Expression study of JS-1 and JS-2 in human ESCC cell lines and tissue specimens. Semi-quantitative RT-PCR analysis for JS-1 and JS-2 was performed on ESCC cell lines and the results are shown in **(A)** and **(B)** respectively. NE3 represents non-tumor epithelial cell line and β-actin expression was used as an internal control. ESCC cell lines KYSE 30, 450, 510, HKESC-3 and SLMT-1 show overexpression of JS-1 and only KYSE 180 and SLMT-1 overexpress JS-2. JS-1 and JS-2 expression in human ESCC specimens are shown in **(C)** and **(D)** respectively. Details of the ESCC cases studied are described in Table 5. N : corresponding non-tumor tissue; T: ESCC tissue. Expression analysis of JS-1 and JS-2 in multiple tissue cDNA (MTC) panel is shown in **(E)**. All the organs studied show JS-1 expression with relatively lower level of expression found in the esophagus. JS-2 was only expressed in the stomach, ileocecum, jejunum and expression was relatively lower in the rectum. Lanes 1-10: 1, esophagus; 2, stomach; 3, duodenum; 4, ileocecum; 5, jejunum; 6, colon (ascending); 7, colon (descending); 8, colon (transverse); 9, rectum; 10, liver.


A
NE3
KYSE 30
NE3
KYSE 70
NE3
KYSE 140
NE3
KYSE 150
NE3
KYSE 180
NE3
KYSE 410
NE3
KYSE 450
NE3
KYSE 510
NE3
KYSE 520
NE3
HKESC-3
NE3
SLMT-1
β-actin (540bp)
JS-1 (327bp)
B
NE3
KYSE 30
NE3
KYSE 70
NE3
KYSE 140
NE3
KYSE 150
NE3
KYSE 180
NE3
KYSE 410
NE3
KYSE 450
NE3
KYSE 510
NE3
KYSE 520
NE3
HKESC-3
NE3
SLMT-1
β-actin (540bp)
JS-2 (422bp)

C


Patient no. 1
N
T
β-actin (540bp)
JS-1 (327bp)
Patient no. 2
Patient no. 3
Patient no. 4
Patient no. 5
Patient no. 6
Patient no. 7
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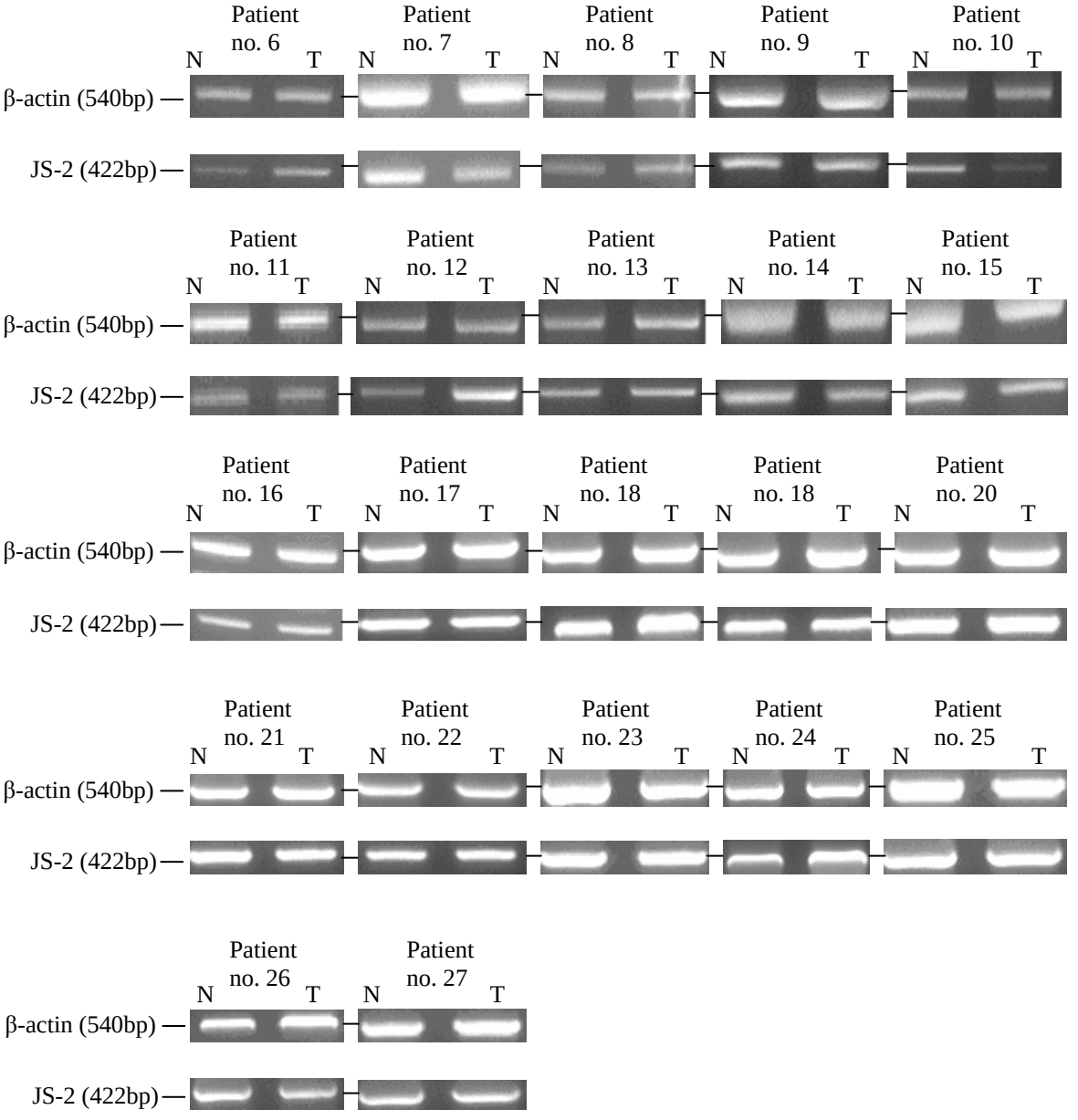
Patient no. 27

D


Patient no. 6
Patient no. 7
Patient no. 8
Patient no. 9
Patient no. 10
N T N T N T N T N T
β-actin (540bp)
JS-2 (422bp)
Patient no. 11
Patient no. 12
Patient no. 13
Patient no. 14
Patient no. 15
N T N T N T N T N T
β-actin (540bp)
JS-2 (422bp)
Patient no. 16
Patient no. 17
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Patient no. 20
N T N T N T N T N T
β-actin (540bp)
JS-2 (422bp)
Patient no. 21
Patient no. 22
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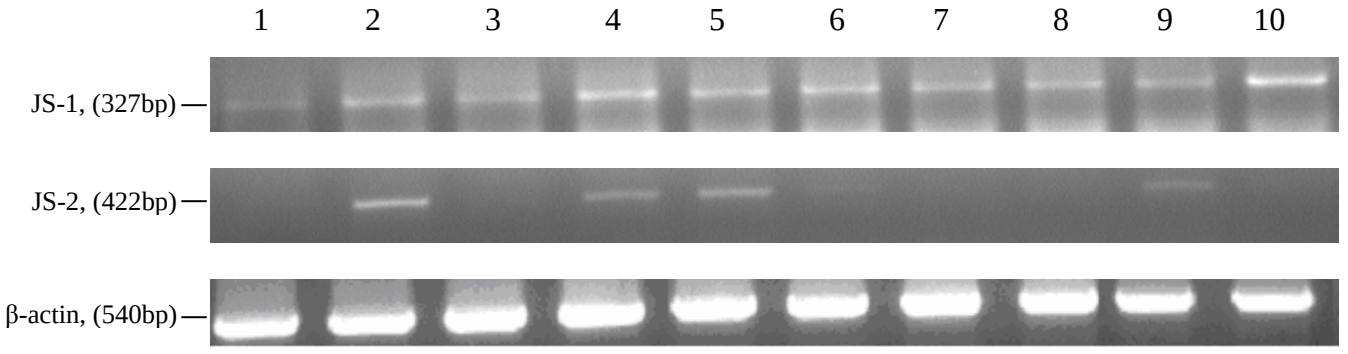
N T N T N T N T N T
β-actin (540bp)
JS-2 (422bp)
Patient no. 26
Patient no. 27
N T N T
β-actin (540bp)
JS-2 (422bp)

E



ESCC cell lines	JS-1[a]	JS-1 expression	JS-2[b]	JS-2 expression
KYSE 30	1.5	↑	1.2	-
KYSE 70	1.2	-	0.9	-
KYSE 140	1.0	-	0.9	-
KYSE 150	0.6	↓	0.6	↓
KYSE 180	1.1	-	2.2	↑
KYSE 410	0.5	↓	0.9	-
KYSE 450	2.8	↑	1.1	-
KYSE 510	1.6	↑	1.1	-
KYSE 520	0.9	-	0.7	↓
HKESC-3	2.2	↑	0.9	-
SLMT-1	1.4	↑	2.2	↑

Table 3. Comparison of JS-1 and JS-2 expression in ESCC cell lines by semi-quantitaive RT-PCR. JS-1[a]: Ratio of [(JS-1 /β-actin, ESCC cell line)] / [(JS-1 /β-actin in NE3 cell line)]; JS-2[b]: Ratio of [(JS-2 /β-actin, ESCC cell line)] / [(JS-2 /β-actin in NE3 cell line); (↑) upregulation; (↓) downregulation; (-) no change in expression.

Patient no.	JS-1[a]	JS-2[b]
1	4.6	N.D.
2	4.5	N.D.
3	3.1	N.D.
4	4.7	N.D.
5	0.9	N.D.
6	0.3	1.9
7	1.7	0.7
8	3.7	0.9
9	4.3	0.8
10	3.1	0.2
11	3.1	0.7
12	2.0	1.6
13	1.9	0.9
14	0.2	0.9
15	1.0	0.8
16	0.9	0.9
17	1.4	0.9
18	1.2	3.0
19	1.1	0.9
20	0.5	0.9
21	0.4	0.9
22	0.5	1.0
23	1.8	0.9
24	1.0	1.2
25	2.5	0.9
26	3.7	0.9
27	0.6	0.9

Table 4. Comparison of JS-1 and JS-2 expression in ESCC specimens and their corresponding non-tumor tissues by semi-quantitative RT-PCR. JS-1[a]: Ratio of [(JS-1 /β-actin, tumor)] / [(JS-1 /β-actin in paired non-tumor)]; JS-2[b]: Ratio of [(JS-2 /β-actin, tumor)] / [(JS-2 /β-actin in paired non-tumor); N.D., RT-PCR analysis for expression was not done because of insufficient tissue availability.

Patient no.	Age/sex	Histopathological type	Tumor stage	Histological stage	JS-1 expression	JS-2 expression
1	49/M	SCC(M)	$T_4N_1M_1$	IV	↑	N.D.
2	57/M	SCC(W)	$T_3N_1M_0$	III	↑	N.D.
3	60/M	SCC(M)	$T_3N_1M_1$	IV	↑	N.D.
4	73/M	SCC(P)	$T_3N_1M_0$	III	↑	N.D.
5	74/M	SCC(M)	$T_3N_0M_0$	II	-	N.D.
6	47/M	SCC(M)	$T_3N_1M_0$	III	↓	↑
7	59/M	SCC(M)	$T_3N_0M_0$	II	↑	↓
8	68/M	SCC(M)	$T_3N_0M_0$	II	↑	-
9	45/M	SCC(P)	$T_4N_1M_0$	III	↑	-
10	74/M	SCC(M)	$T_3N_0M_0$	II	↑	↓
11	66/M	SCC(M)	$T_3N_1M_0$	III	↑	↓
12	71/M	D	$T_0N_0M_0$	N.A.	↑	↑
13	58/M	SCC(M)	$T_3N_0M_0$	II	↑	-
14	74/M	N.T./(Post CTRT)	N.T.	N.A.	↓	-
15	57/M	SCC(W)	$T_4N_0M_0$	III	-	-
16	63/M	MEC	$T_3N_1M_0$	III	-	-
17	57/M	SCC(M)	$T_3N_0M_0$	II	↑	-
18	77/M	SCC(W)	$T_4N_1M_0$	III	-	↑
19	70/M	SCC(M)	$T_3N_0M1_b$	IV	-	-
20	71/M	SCC(P)	$T_3N_0M_0$	II	↓	-
21	74/M	SCC(M)	$T_3N_1M_0$	III	↓	-
22	66/M	SCC(W)	$T_3N_0M_{1a}$	IV	↓	-
23	76/M	SCC(M)	$T_3N_0M_0$	II	↑	-
24	57/M	SCC(W)	$T_3N_0M_0$	II	-	-
25	70/M	SCC(W)	$T_3N_0M_0$	II	↑	-
26	60/M	SCC(P)	$T_3N_1M_0$	III	↑	-
27	68/M	SCC(P)	$T_3N_1M_0$	III	↓	-

Table 5. Summary of JS-1 and JS-2 expression studies and clinical data of ESCC patients studied. SCC, squamous cell carcinoma; MEC, mucoepidermoid carcinoma; Post CTRT, patient received chemoradiotherapy before operation; (D), dysplasia; (W), well differentiated type of tumor; (M), moderately differentiated type of tumor; (P), poorly differentiated type of tumor; N.D., RT-PCR analysis for expression was not done because of insufficient tissue availability; N.A., not applicable; N.T., non-tumor; (↑) upregulation; (↓) down regulation; (-) no change in expression.

3.3 Analysis and conclusion

This part of the study confirmed the identities and investigated the expression of two novel genes named as JS-1 and JS-2, located on chromosome region 5p15.2 and upstream to delta catenin gene.

The results of the 3' and 5' RACE products of JS-1 and JS-2 genes confirmed that the transcripts studied were identical to that of the assembled sequence previously published in the GenBank (LOC134147 and LOC134145 for JS-1 and JS-2 genes respectively). The start codons for JS-1 and JS-2 are embedded in a kozak sequence to assist in efficient initiation of translation (Kozak M, 1987). The PolyA signal, required for mRNA stability, mRNA export from the nucleus to the cytoplasm, translation and initiation (Zarudnaya et al., 2003), are different for JS-1 (AATAAA / AAUAAA hexamer) and JS-2 (ACTAAA / ACUAAA hexamer). The two common polyA signals in human mature mRNAs are the AAUAAA and AUUAAA hexamers, present in 73% of known mRNAs. However, ten single-base variants of AAUAAA hexamer have been identified with highly significant occurrence rate (Beaudoing *et al.*, 2000). Taken together, AAUAAA and AUUAAA polyA signal hexamers are not universal. JS-2 transcript contains ACTAAA hexamer (ACUAAA) as the polyA signal, which is a variant of AAUAAA hexamer.

Following identification of JS-1 and JS-2 transcripts from the RACE study, the level of JS-1 and JS-2 expression in ESCC cell lines and specimens were studied by semi-quantitative RT-PCR. JS-1 was overexpressed in 45% (5/11) of ESCC cell lines and in 56% (15/27) of ESCC tissues. Several other genes implicated in ESCC carcinogenesis have also been reported to have high levels of overexpression e.g cyclin D1 63% (Adelaide *et al.,* 1995), EphA2 50% (Miyazaki *et al.,* 2003a), stromelysin-2 74% (Mathew *et al.,* 2002), fascin 60% (Hashimoto *et al.,* 2005), Aurora-A 67.5% (Tong *et al.,* 2004), and ETS2 (erythroblastosis virus oncogene homolog 2) expression was upregulated in 75.7% of ESCC tissues examined (Li *et al.,* 2003). Therefore JS-1 overexpression may be significant in ESCC. Correlation between JS-1 overexpression and clinicopathological features of ESCC cases studied show that of the fifteen ESCC cases overexpressing JS-1, 47% (7/15) were of tumor stage II, 33% (5/15) were of tumor stage III, 13% (2/15) were of tumor stage IV and 7% (1/15) were of dysplastic type. Higher percentage of genetic alterations in stage III than in stage IV ESCC were also reported in cyclin D1 (Itami *et al*., 1999) and hst-1 (Chikuba *et al*., 1995) amplification. Ebihara *et al.,* (2004) reported higher number of ESCC patients with E2F1 overexpression in stage II than in the later stages. Ito *et al.,* (2003) also reported higher percentage of TSLC1 overexpression in stage III ESCC than in stage IV. According to Table 5, 60% (9/15) of ESCC cases overexpressing JS-1 were of moderately differentiated histopathological type.

In contrast, JS-2 was overexpressed in only 18% (2/11) of ESCC cell lines and 14% (3/22) of ESCC specimens. Of the three ESCC cases overexpressing JS-2, two were of tumor stage III and one with no staging. This may suggest overexpression of JS-2 may not play a significant role in ESCC on its own. The ESCC specimens were provided by the Department of Surgery of Queen Mary Hospital in Hong Kong and our limited accessibility to surgical specimens restricted the RT-PCR expression analysis to a limited pool with the distribution of various histological stages and histopathological types as included in the present study. Most specimens were of tumor stages II and III and moderately differentiated histopathological type because in Hong Kong most patients are diagnosed at tumor stage II and III and moderately differentiated squamous cell carcinomas are the most common (Lam KY and Ma L, 1997a). A larger study with equal distribution of histological stages and histopathological types will be more accurate in analyzing the expression of JS-1 and JS-2 in the development of ESCC.

To conclude, a novel gene JS-1 was shown to be overexpressed in 45% (5/11) of ESCC cell lines and in 56% (15/27) of ESCC specimens, suggesting its possible importance and involvement in ESCC development. Another novel gene JS-2 was only overexpressed in 18% (2/11) of ESCC cell lines and in 14% (3/22) of ESCC specimens and it may only be involved to a lesser extent in this disease. The next

question addressed was whether overexpression of JS-1 and JS-2 in normal NIH 3T3 cells induces transformation. The following study will attempt to answer this.

CHAPTER 4 TRANSFORMING AND TUMORIGENIC PROPERTIES OF NIH 3T3 CELLS TRANSFECTED WITH JS-1 AND JS-2

4.1 Selection of samples and controls

NIH 3T3 cells were used to study the cell-transforming effects of JS-1 and JS-2 overexpression. NIH 3T3 cells transfected with a) pcDNA3.1(-) vector only (named as 3T3/vec); b) pcDNA3.1-H-*ras*V12 construct (named as 3T3/ras); c) JS-1 (named as 3T3/JS-1); and d) JS-2 (named as 3T3/JS-2) were selected in 300μg/ml G418 antibiotic for 14 days following transfection, as described in section 2.3.5.2.

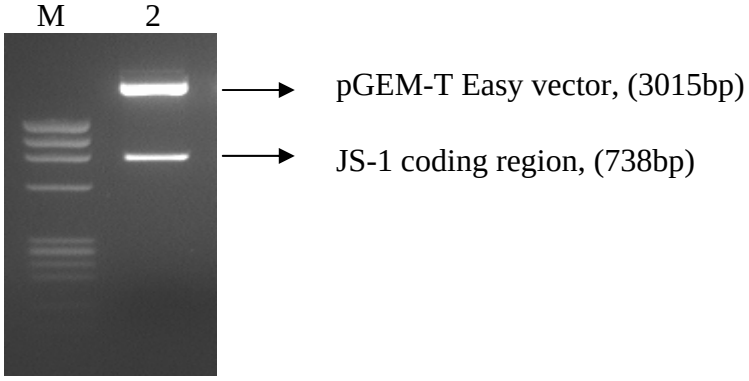
Parental NIH 3T3 cells and 3T3/vec cells served as negative controls and 3T3/ras cells served as a positive control. pcDNA3.1-H-*ras*V12 construct contains an oncogenic ras with glycine-to-valine mutation at residue 12 (Jin *et al.*, 2003; Yang *et al.*, 2000).

4.2 Results

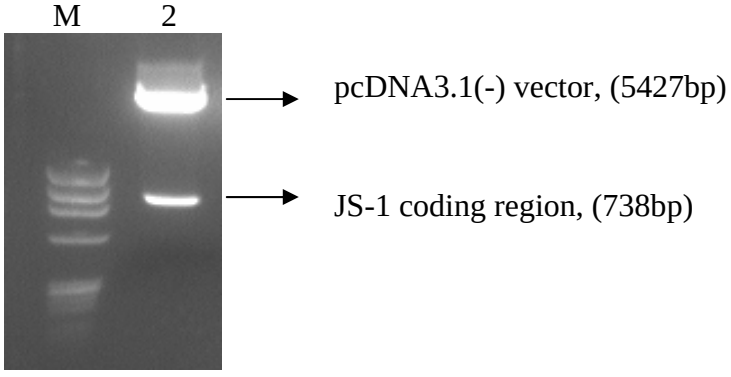
4.2.1 Cloning of JS-1 and JS-2 coding sequence into expression vector for transfection studies

Coding regions of JS-1 and JS-2 genes were produced by PCR as described in section 2.3.5.1 and were first cloned into pGEM-T Easy vector followed by subcloning into pcDNA3.1(+/-) vectors. Transfection studies were conducted on NIH 3T3 cells following the successful cloning.

Figure 9A confirms the presence of JS-1 coding region in pGEM-T Easy vector and in pcDNA3.1(-) vector. Figure 9B confirms the presence of JS-2 coding region in pGEM-T Easy vector and in pcDNA3.1(+) vector. DNA sequencing verified the sequences of JS-1 and JS-2 coding regions and also their orientation with respect to the transcription start site in pcDNA3.1(+/-) vectors.

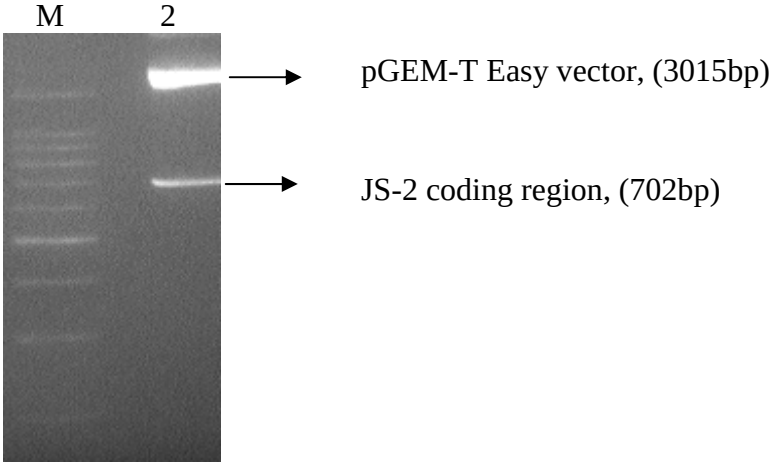


a. The JS-1 coding region cloned into pGEM-T Easy vector was confirmed by NotI restriction enzyme digestion (lane 2) and DNA sequencing.

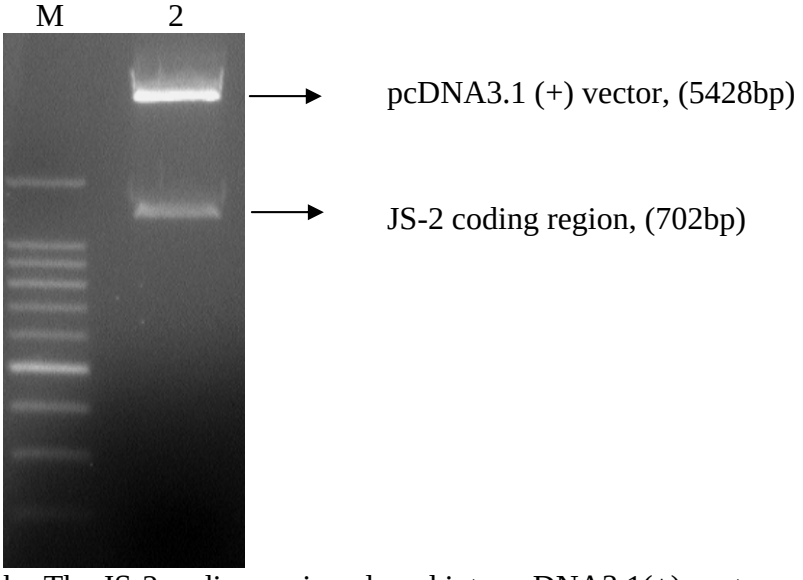


b. The JS-1 coding region cloned into pcDNA3.1(-) vector was confirmed by BamHI and ApaI restriction enzyme digestion (lane 2) and DNA sequencing.

Figure 9A. Confirmation of the presence of JS-1 coding region in a) pGEM-T Easy vector and in b) pcDNA3.1(-) vector. M: molecular size marker (ϕ174/Hae III cut).



a.The JS-2 coding region cloned into pGEM-T Easy vector was verified by EcoRI restriction enzyme digestion (lane 2) and DNA sequencing.



b. The JS-2 coding region cloned into pcDNA3.1(+) vector was verified by EcoRI restriction enzyme digestion (lane 2) and DNA sequencing.

Figure 9B. Confimration of the presence of JS-2 coding region in a) pGEM-T Easy vector and in b) pcDNA3.1(+) vector. M: molecular size marker (100bp DNA ladder).

4.2.2 Soft agar assay, growth curve and foci formation assay

In order to study the roles of JS-1 and JS-2 overexpression in relation to their transforming potentials in normal cells, *in vitro* studies of colony formation in soft agar, foci formation, and growth rate of transfected cells were performed. 3T3/JS-1 cells and 3T3/ras cells formed colonies in soft agar but colony formation was not observed in parental NIH 3T3 cells, 3T3/vec cells and 3T3/JS-2 cells (Figure 9C). Thus hereafter, we focused on the overexpression study of JS-1 and subsequent tests were only performed on JS-1. To determine whether JS-1 overexpression affected the growth of transfected cells, parental NIH 3T3 cells, 3T3/vec cells, 3T3/JS-1 cells and 3T3/ras cells were seeded in culture flasks containing DMEM medium supplemented with 10% FBS and with or without addition of 300μg/ml G418 antibiotic. According to the growth curve (Figure 9D) 3T3/JS-1 cells proliferated as rapidly as the positive control cells (3T3/ras) and the doubling time was reduced from 24.1hr in parental NIH 3T3 cells to 17.2hr in 3T3/JS-1 cells. Moreover, 3T3/JS-1 cells and 3T3/ras cells lost their contact inhibition when they reached confluent growth and formed detectable foci (Figure 9E). In contrast, the parental NIH 3T3 cells and 3T3/vec cells exhibited contact inhibition and did not form detectable foci.

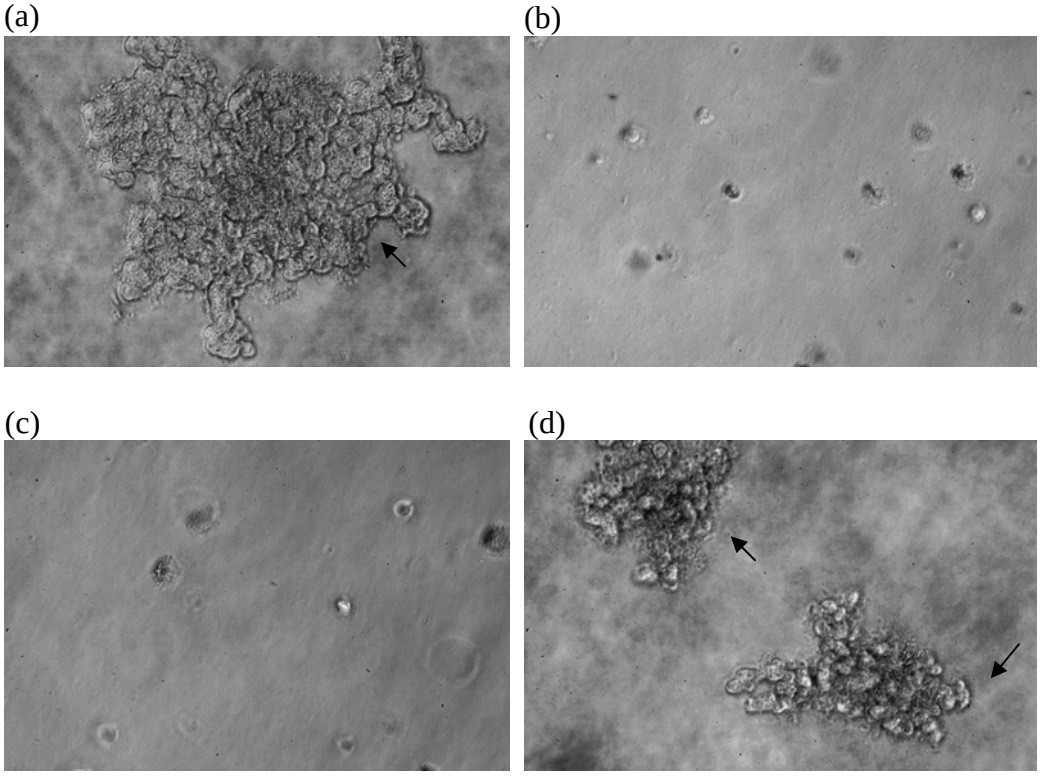


Figure 9C. Anchorage-independent growth of NIH 3T3 cells transfected with (a) JS-1 (b) JS-2 (c) pcDNA3.1(-) vector only and (d) pcDNA3.1-H-*ras*V12. Colonies (arrows) were formed in NIH 3T3 cells transfected with JS-1 and pcDNA3.1-H-*ras*V12 but not in cells transfected with JS-2 and mock vector. Photographs were taken after 16 days of cell growth in soft agar. Original magnification: x200.

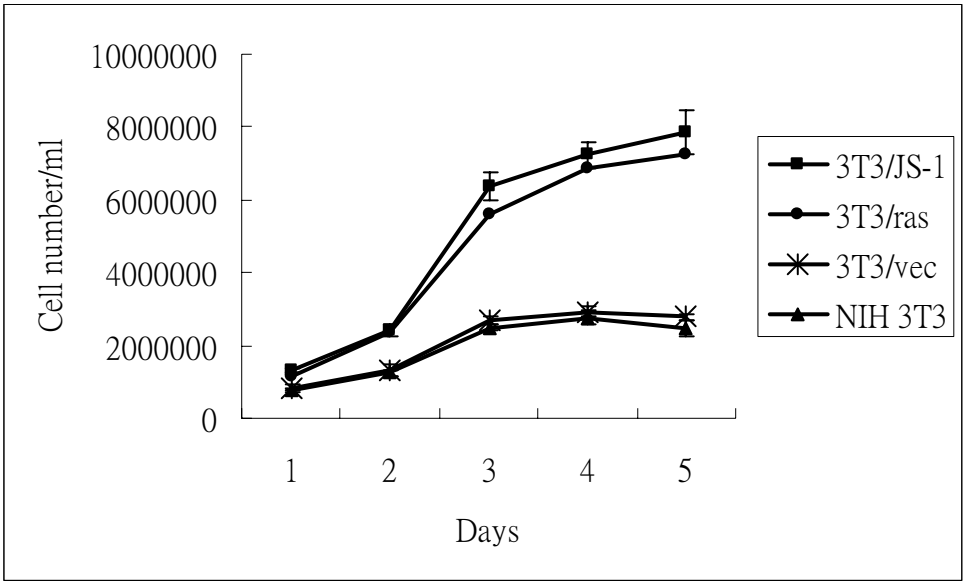


Figure 9D. Growth curves of parental NIH 3T3 cells, 3T3/JS-1 cells, 3T3/vec cells and 3T3/ras cells. Cells were grown under conditions as described in Materials and Methods. Cells were counted on indicated days with a haemocytometer. All results are the means of triplicate experiments and the standard deviations are indicated by the standard bars.

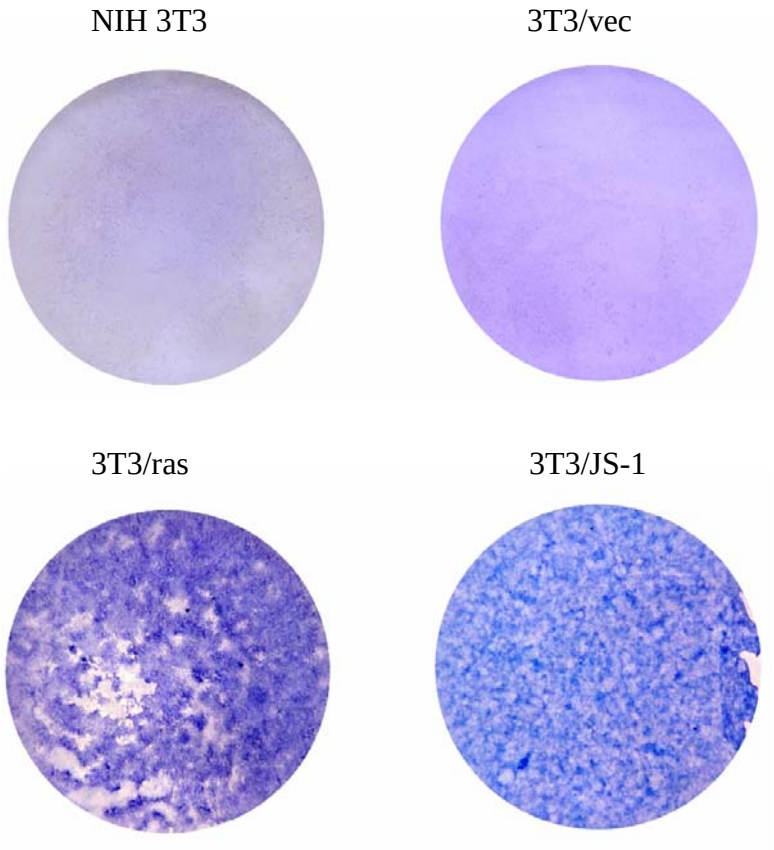


Figure 9E. Anchorage-dependent growth assay to show foci formation in parental NIH 3T3 cells, 3T3/vec cells, 3T3/ras cells and 3T3/JS-1 cells at day 14 after seeding of cells. Foci formation could be observed in NIH 3T3 cells transfected with pcDNA3.1-H-*ras*V12 and JS-1 but not in the parental NIH 3T3 cells and those transfected with the mock vector.

4.2.3 Tumorigenicity test

To investigate the tumorigenic potential of JS-1 when it is overexpressed, 3T3/JS-1 cells were subcutaneously injected into five athymic nude mice. 3T3/JS-1 cells induced subcutaneous tumor formation at the site of injection in all the mice tested and 3T3/vec cells did not form any subcutaneous tumor (Figure 10A). A histological analysis of the tumor formed by 3T3/JS-1 cells showed a hypercellular tumor which is composed of spindle tumor-like cells with prominent nucleoli and frequent mitotic figures (Figure 10B). The tumor cells infiltrated through the skeletal muscles. The overall features are those of a malignant tumor. Further study using cytokeratin markers (Mak-6, AE1/3) and desmin is required to determine whether the tumor is spindle cell carcinoma or a sarcoma. To confirm the expression level of JS-1 in the subcutaneous tumors and the transfected cells, RT-PCR was performed on parental NIH 3T3 cells, 3T3/vec cells, 3T3/JS-1 cells and on the subcutaneous tumor to show the expression level of JS-1. The subcutaneous tumor and 3T3/JS-1 cells show overexpression of JS-1 compared with the controls (Figure 10C).

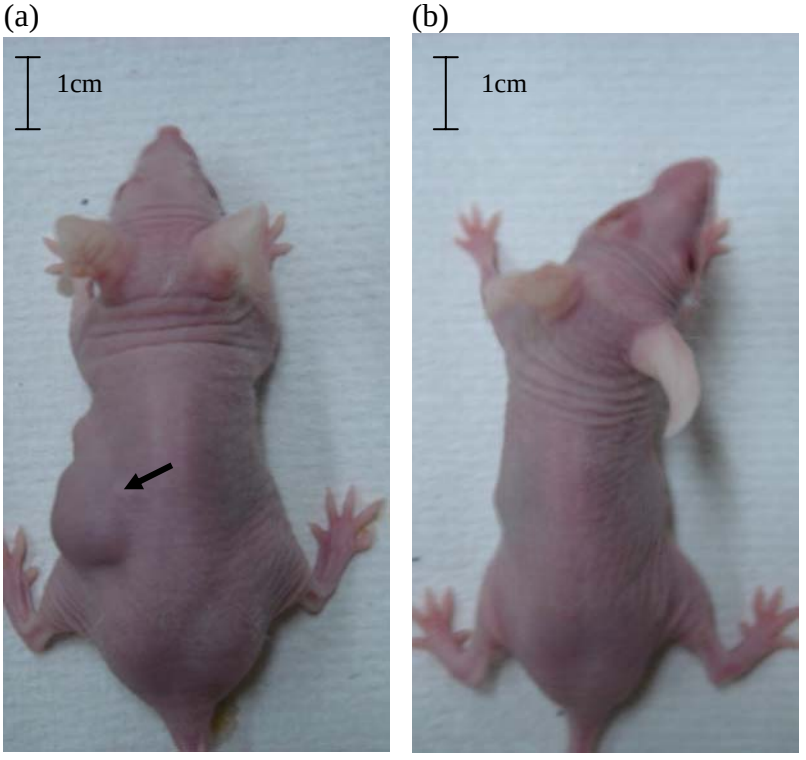


Figure 10A. Tumorigenicity test using athymic nude mice with subcutaneous injection of a) 3T3/JS-1 cells and b) 3T3/vec cells. Subcutaneous formation of tumor mass (arrow) could be observed in the mouse injected with 3T3/JS-1cells but not in the mock control after 30 days of injection.

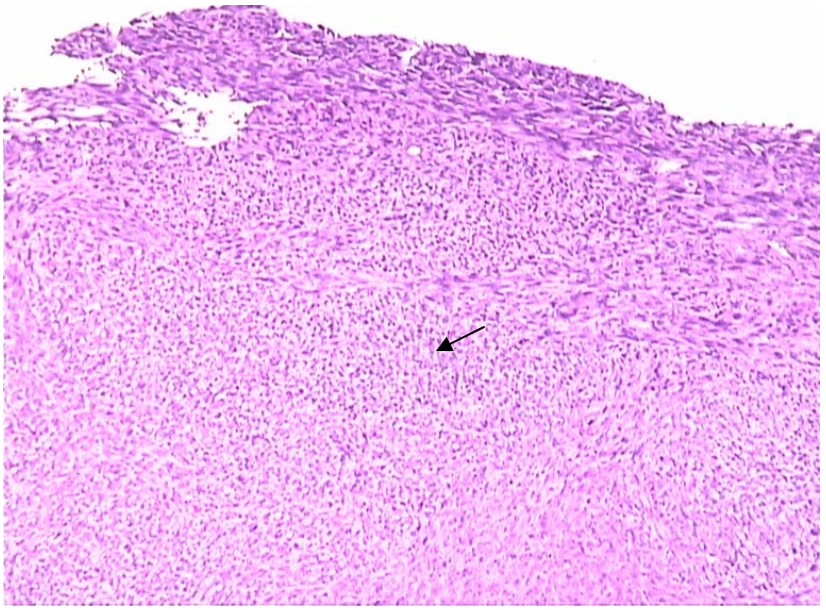


Figure 10B. Histological analysis on the subcutaneous tumor formed in athymic nude mice by the injection of 3T3/JS-1 cells showing the morphology of a malignant tumor (arrow). Hematoxylin & eosin stained; original magnification x200.

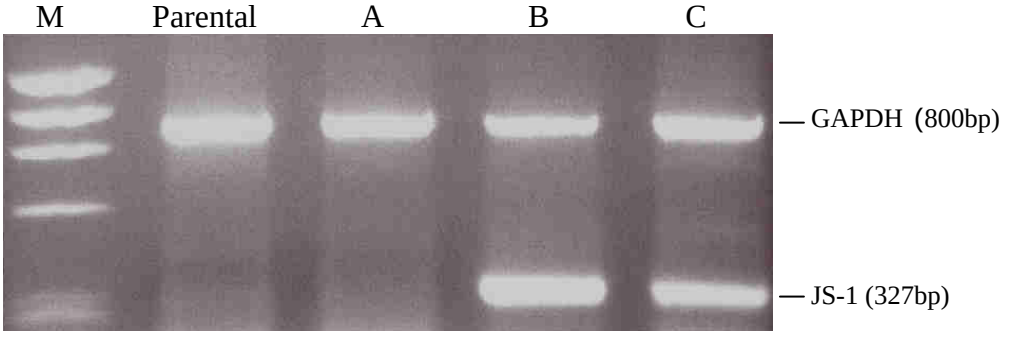


Figure 10C. Semi-quantitative RT-PCR analysis of JS-1 expression in parental NIH 3T3 cells (Parental), NIH 3T3 cells transfected with (A) pcDNA3.1(-) vector only, (B) JS-1, and (C) subcutaneous tumor tissue from athymic nude mouse. GAPDH expression served as the internal control. M: molecular size marker (ϕ174/Hae III cut).

4.3 Analysis and conclusion

This part of the study investigated the transforming potential of JS-1 and JS-2 when they are overexpressed in normal NIH 3T3 cells. Some of the common phenotypic changes *in vitro* for cancer cells undergoing cellular transformation are an increase in growth rate (Wolfgang *et al.*, 2001), formation of foci and anchorage-independent growth (Robert C Millers, 1999). Although the overexpression of JS-1 in NIH 3T3 cells did not lead to an observable change in the cell morphology, its overexpression led to foci formation and induced selective growth advantage in 3T3/JS-1 cells as they proliferated faster than parental NIH 3T3 cells and 3T3/vec cells. Overexpression of JS-1 in anchorage-dependent NIH 3T3 cells also resulted in their anchorage-independent growth, as shown by soft agar assay. Soft agar assay is a stringent assay for detecting malignant transformation of cells and was developed by Hamburger and Salmon (1977). In this assay, each colony observed was composed from a clone of cells that originated from a single cell whose growth was promoted by overexpression of JS-1, as shown by RT-PCR for JS-1 expression (Figure 10C). The results of the *in vitro* study imply that overexpression of JS-1 in normal NIH 3T3 cells elicits cellular transformation.

However, cellular transformation does not always imply acquisition of tumorigenicity (Okamoto *et al.,* 1996; Valverius *et al.,* 1989). To examine if JS-1 overexpression confers tumorigenicity, 3T3/JS-1 cells were transfected into athymic nude mice and tumor formation was observed in all (5/5, 100%) athymic nude mice transfected. The correlation between anchorage-independent growth of cells in semi-solid medium and tumorigenicity was first reported by Freedman *et al*., (1974). Later Shin *et al.,* (1975) showed that the only cellular property repeatedly linked with tumorigenicity in athymic nude mice is the ability of transformed cells to proliferate *in vitro* in the absence of anchorage and Stiles *et al.,* (1976) concluded that tumorigenicity in athymic nude mice is a reliable assay for malignant transformation. Therefore, the overexpression of JS-1 may play an oncogenic role in ESCC but the cellular function(s) of JS-1 in normal cells need to be investigated first before it could be classified as a proto-oncogene. Furthermore, the cause of JS-1 overexpression remains to be investigated. Several genes overexpressed and implicated in various cancers have also shown to induce tumors in athymic nude mice. Overexpression of SKP2 (S-phase kinase-associated protein 2) results in colony formation in soft agar and tumor formation in athymic nude mice (Gstaiger *et al.,* 2001) and it is overexpressed in ESCC (Fukuchi *et al.,* 2004), lymphomas (Latres *et al.,* 2001), oral squamous cell carcinoma (Gstaiger *et al.,* 2001), colorectal carcinomas (Hershko *et al.,* 2001) and prostate cancer (Carrano and Pagano, 2001).

Overexpression of MDM2 has also shown to confer tumorigenicity in athymic nude mice (Zhang *et al.,* 2005; Fakharzadeh *et al.,* 1991) and it is overexpressed in 22% of esophageal cancers (Morgan *et al.,* 1999). JS-2 overexpression in NIH 3T3 cells did not induce colony formation in soft agar assay suggesting it does not stimulate cellular transformation.

To conclude, JS-1 overexpression induced cellular transformation on NIH 3T3 cells through four observations: selective growth advantage, formation of foci on a monolayer of cells, anchorage-independent growth in soft agar and tumor formation in athymic nude mice.

CHAPTER 5 BIOINFORMATICS SEARCH ON JS-1 AND JS-2

5.1 Bioinformatics search

JS-1 was predicted to encode a novel protein of 245 amino acids that could not be matched with any known protein sequences in SwissProt using the BLAST search program that is available in the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The amino acid sequence also contained no well-characterized functional motifs when it was matched against the e-Motif search program that is available from Stanford University or the Motif Scan program available in the Institute for Chemical Research, Kyoto University which matches against the PROSITE patterns. When the JS-1 amino acid sequence was scanned against the PROSITE database using the ScanProsite program that is available in the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics, JS-1 protein consisted of two casein kinase II phosphorylation (CKII) sites at the amino acid residues 37-40 (S-P-V-D) and 223-226 (S-P-A-D). CKII is a multifunctional protein kinase that has been implicated in a variety of cellular processes and functions, including mitosis and cellular transformation. Several nuclear proteins, enzymes and transcriptions factors serve as substrates for CKII (Tuazon and Traugh,

1991). One protein kinase C phosphorylation site (PKC) was observed at amino acid residues 191-193 (T-Q-K). PKC mediates most signal transduction in eukaryotic cells and they also maintain cellular processes including metabolism, transcription, cell cycle proliferation, apoptosis and differentiation (Keenan and Kelleher, 1998). One tyrosine kinase phosphorylation (TK) site was also observed in JS-1 at the amino acid residues 159-165 (K-D-S-E-D-I-Y). Focal adhesion kinase (FAK) and EphA2 are examples of tyrosine kinases reported to be implicated in ESCC. Overexpression of FAK has been linked to cell differentiation, tumor invasiveness, lymph node metastasis and poor prognosis (Miyazaki *et al.,* 2003a). EphA2 overexpression has also been related to poor prognosis in ESCC (Miyazaki *et al.,* 2003b). One tyrosine sulfation site was observed at amino acid residues 158-172 (V-K-D-S-E-D-I-Y-N-L-K-N-P-T-L). Tyrosine sulfation is a post-translational modification of tyrosines that go through the trans-Golgi network and that are important in the immune system (Bernimoulin *et al.,* 2003; Lin *et al.,* 2003). In addition, JS-1 also showed two N-myristoylation sites at amino acid residues 68-73 (G-N-G-Y-T-T) and 209-214 (G-Q-T-H-G-F). N-myristoyltransferase, which catalyzes N-myristoylation, is highly expressed in gall bladder carcinoma (Rajala *et al.,* 2000) and colon cancer (Magnuson *et al.,* 1995). Many known myristoylated proteins appear to be kinases (King *et al.,* 1993). Tumorigenic role of JS-1 overexpression suggests further investigations into its relationship with TK, PKC, CKII, tyrosine sulfation and N-myristoylation. JS-2 was

predicted to encode a novel protein of 233 amino acids that could not be matched with any known protein sequences in SwissProt. ScanProsite analysis found that JS-2 protein was found to contain 2 casein kinase II phosphorylation sites at amino acid residues 11-14 (T-L-K-E) and 91-94 (S-L-V-D), four protein kinase C phosphorylation sites at amino acid residues 11-13 (T-L-K), 74-76 (T-M-K), 138-140 (S-A-K) and 213-215 (T-F-R)], four N-myristoylation sites at amino acid residues 42-47 (G-L-V-G-G-T), 46-51 (G-T-L-V-A-V), 96-101 (G-S-G-D-G-R) and 134-139 (G-V-H-G-S-A), and one cAMP- and cGMP-dependent protein kinase phosphorylation site at amino acid residues 88-91 (R-R-G-S).

No homology was found for JS-1 and JS-2 with known functional genes. However, JS-1 shares 82% homology at the cDNA level to a mouse hypothetical gene, 2310016A09Rik. According to the SOURCE database, available from Stanford University, 2310016A09Rik gene is predicted to have catalytic and hydrolytic activities and is expressed in liver, kidney, whole eye (retinal degeneration), pituitary gland, heart, pancreatic islet and testis. Table 6 summarizes the bioinformatics analysis on JS-1 and JS-2 transcripts and proteins.

To conclude, bioinformatics analysis displayed that JS-1 and JS-2 proteins are composed of many types of domains including some important kinase

phosphorylation sites. Thus, it can be speculated that JS-1 and JS-2 could be involved in some physiological processes through these domains and the precise functions of JS-1 and JS-2 need to be investigated.

Search program	JS-1	JS-2
NCBI-BLAST search (http://www.ncbi.nih.gov/BLAST/)	No homology to known functional genes	No homology to known functional genes
ScanProsite search (http://au.expasy.org/)	• two casein kinase II phosphorylation sites • one protein kinase C phosphorylation site • one tyrosine kinase phosphorylation site • one tyrosine sulfation site • two N-myristoylation sites	• two casein kinase II phosphorylation sites • four protein kinase C phosphorylation sites • four N-myristoylation sites •one cAMP- and cGMP-dependent protein kinase phosphorylation site

Table 6. Bioinformatics analysis on JS-1 and JS-2 transcripts and proteins.

CHAPTER 6 DISCUSSION

ESCC is an aggressive cancer that is characterized by a high mortality rate and geographic differences in incidence (Si *et al.,* 2003). Despite advances in multimodality treatment, the five-year survival rate of ESCC remains below 10% (Kwong *et al.,* 2004b). Identification of molecular therapeutic targets may be important in improving the survival of patients. Genetic alterations involved in the development or progression of ESCC have been studied (Lam KY 2000; Tang *et al.,* 2001b; Metzger *et al.,* 2004) but a complete picture about the molecular pathogenesis of ESCC is still largely not known. Thus, it is important to identify novel genes associated with ESCC and understand the underlying mechanisms of this disease.

The completion of the human genome project has resulted in a vast amount of genomic information and the roles of many identified genes remain unknown. This study looked into the expression of two novel genes, JS-1 and JS-2, located in chromosome region 5p15.2, which are upstream to delta catenin gene and have not been studied before about their roles in the molecular pathogenesis of ESCC. The complete cDNA sequence of JS-1 and JS-2 was determined by 3' and 5' RACE study and the expression pattern of JS-1 and JS-2 was examined by RT-PCR, which

is a fast and convenient method for gene expression study. JS-1 was overexpressed in a high percentage of ESCC cell lines (5/11, 45%) and specimens (15/27, 56%), suggesting its possible importance in ESCC development. However, abnormality in gene expression does not always imply that a gene causes cancer (Shastry B.S., 1995), but the overexpression of JS-1 demonstrated oncogenic potential by transforming NIH 3T3 cells as seen by colony formation in soft agar assay and tumor formation in athymic nude mice. The proliferative advantage acquired from JS-1 overexpression in NIH 3T3 cells was sufficient to enable a generation of vast cell populations that constituted the tumors in the athymic nude mice. Thus, the abnormality of JS-1 overexpression may play a role in ESCC carcinogenesis. It is not known how JS-1 overexpression confers tumorigenicity and the cellular function(s) of JS-1 in normal cells need to be investigated first before it could be classified as a proto-oncogene. Han *et al.,* (1993) and Guadagno *et al.,* (1991) suggested that adhesion can also be viewed as a cell cycle regulator and that in NIH 3T3 cells the part of the cell cycle that requires adhesion is transition from G1 to S phase of the cell cycle. In the absence of adhesion, anchorage-dependent cells arrest at the G1/S phase i.e. before DNA synthesis but transformed cells progress through the S phase of the cell cycle (Guadagno *et al.,* 1991). JS-1 may act alone or indirectly by altering the activities of some transcriptional activators, which in turn regulate many target genes or cellular factors involved in cell proliferation and

tumorigenesis. Thus, the knowledge of the mechanisms of tumorigenesis of the single-gene overexpression of JS-1 awaits further investigation. One example of gene involved in the progression through the G1/S phase transition is cyclin D1 and it is also implicated in ESCC (Nakagawa *et al.,* 1995). Zhu *et al.,* (1996) reported that adhesion is important for expression of cyclin D1 in NIH 3T3 cells and human fibroblasts and that its forced expression rescues retinoblastoma phosphorylation and entry into S phase of the cell cycle. Therefore the transforming effects of oncogenes, mutation or deletion in tumor suppressor genes and DNA viruses could be linked to evasion of cell adhesion affecting cdk complexes that guide cells through G1 and S phase of the cell cycle (Zhu *et al.,* 1996; Hunter T and Pines J, 1994). Identification of a single gene that results in tumorigenesis have also been reported e.g. ras (Giehl K, 2005), MDM2 (Zhang *et al.,* 2005), SKP2 (Gstaiger *et al.,* 2001) and glucose-6-phosphate dehydrogenase (Kuo *et al.,* 2000).

Of the fifteen ESCC cases overexpressing JS-1, 47% (7/15) of ESCC cases were of tumor stage II and 33% (5/15) of ESCC cases were of tumor stage III, suggesting upregulation of JS-1 in the middle stage of malignant transformation of ESCC. A large scale study is needed to correlate JS-1 expression to prognosis of this disease, which remains poor. Various attempts have been made to investigate the relationship between certain molecular markers and the clinical course of ESCC but

the results are obscure. Several molecular factors proposed as prognostic indicators of ESCC include, RCAS1 (Kato *et al.*, 2005), Mina53 (Tsuneoka *et al.*, 2004), mdm2 (Ikeguchi *et al.*, 2002), Bax (Ikeguchi *et al.*, 2001b), nm23-H1 (Iizuka *et al.*, 1999), cyclin D1 (Itami *et al.*, 1999), and p53 (Wang et al., 1994). In contrast, JS-2 gene was overexpressed in only 18% (2/11) of ESCC cell lines and 14% (3/22) of ESCC specimens, and its overexpression in NIH 3T3 cells did not induce cellular transformation. Although it may not play a role in ESCC on its own but as a novel gene with no known function(s), it will be useful to identify its role in normal cells in future study.

To understand the mechanism of JS-1 upregulation, our research group investigated mutation and polymorphism in the coding region of JS-1 in nine ESCC cell lines (SLMT-1, HKESC-3 and KYSE 30, 150, 180, 410, 450, 510 and 520) and in six randomly selected ESCC specimens. No mutation was detected (Au Ho Wah, 2004; Li Wing Yan, 2004; Tang Wing Ka, 2004) and further studies are needed to understand the cause of JS-1 overexpression. Several factors other than mutation can lead to overexpression of genes e.g. hypomethylation (Sato *et al.*, 2003), factors affecting cis and trans acting elements (Chung *et al.*, 1986) and amplification leading to overexpression is another example of dys-regulation at the genomic level (Sager R, 1997).

Identification and characterization of genes whose expression is dramatically altered in tumor verses normal epithelium may be important in understanding the genetic events underlying cancer development. In the last couple of years, many novel genes have been identified which may play a role in the carcinogenesis of ESCC e.g. ECRG4 (Yue et *al.,* 2003), EC97 (Lu *et al.,* 2003), NMES1 (Zhou *et al.,* 2002), hRFI (Sasaki *et al.,* 2002), GASC1 (Yang *et al.,* 2000) and C1orf10 (Xu *et al.,* 2000). RT-PCR studies and Northern blot analysis have shown that all these genes have dys-regulated expression at the transcriptional level in ESCC cell lines and/or ESCC tissues. Our present study also provides evidence of the involvement of a novel gene JS-1 located on chromosome 5p in the molecular carcinogenesis of ESCC. To date, five known genes located on chromosome 5p have been implicated in ESCC. These genes are the telomerase reverse transcriptase gene, in chromosome region 5p15.33 (Yu *et al.,* 2004; Shen *et al.,* 2003; Tominaga *et al.,* 2002), uracil-DNA glycosylase 2 gene, in chromosome region 5p15.2-5p13.1 (Xiong *et al.,* 2002), SKP2 gene, in chromosome region 5p13 (Fukuchi *et al.,* 2004) and complement component 6 and component 7 genes, in chromosome region 5p13 (Oka *et al.,* 2001). This suggests that genes located on chromosome 5p may be significant in the tumorigenesis of the esophagus and JS-1 was identified from the present study as being one of them. Thus, this region warrants further studies for its role in the development of ESCC.

Amplification of 5p is of particular importance because other than in ESCC, genomic alterations in this region have also been implicated in various carcinomas including breast (Forozan *et al.,* 2000), head and neck (Hashimoto *et al.,* 2000), lung (Balsara *et al.,* 1997), bladder (Voorter *et al.,* 1995), and cervix (Heselmeyer *et al.,* 1997). The combined evidence suggests that this region may harbour oncogenes implicated in different neoplasms. Since ESCC was used in the present study as a target to study JS-1 and JS-2 expression, it will also be useful to study their expression in other cancers. Therefore, it will be important to explore the pathways JS-1 plays a part in and to investigate its interactions with other genes in order to understand the roles of JS-1 in cancer formation.

No homology was found between JS-1 and JS-2 with known functional genes. SwissProt analysis of JS-1 revealed important kinase phosphorylation sites for protein kinase C (PKC), casein kinase II (CKII) and tyrosine kinase (TK). JS-1 could be involved in some physiological processes through these domains. The finding of the tumorigenic role of JS-1 when overexpressed suggests further investigations of the relationship between PKC, CKII, TK and JS-1 in normal cells and cancer cells. This may perhaps reveal a new mechanism of tumorigenesis in ESCC and other cancers.

In summary, this study has investigated a novel human gene designated as JS-1, whose overexpression and transforming capacity in normal cells may play a role in the molecular pathogenesis of ESCC. To determine the role of JS-1 in tumorigenesis of ESCC, more studies are needed to determine how JS-1 is regulated in ESCC tumors and what physiological function(s) of JS-1 are during the development of human tumors.

SUMMARY OF CONCLUSIONS

The complete cDNA sequences of two novel genes, designated as JS-1 and JS-2, were studied in ESCC in the present project. These novel genes are located in chromosome region 5p15.2 and have not been studied in ESCC before. Their expression in ESCC was investigated and conclusions are as follow:

1) Semi-quantitative RT-PCR study showed that JS-1 was overexpressed in a higher percentage of ESCC cell lines (5/11; 45%) and in 15/27 (56%) of ESCC specimens than JS-2 which was overexpressed in only 18% (2/11) of ESCC cell lines and in 14% (3/22) of ESCC specimens.

2) Based on the clinicopathological features of ESCC cases studied, JS-1 overexpression was more commonly found in tumor stage II (7/15; 47%) and stage III (5/15; 33%) of ESCC cases. Also, JS-1 overexpression was more associated with the development of moderately differentiated esophageal squamous cell carcinoma in the ESCC cases studied (9/15; 60%).

3) Overexpression of JS-1 in normal NIH 3T3 cells resulted in cellular transformation and induced subcutaneous tumor formation in athymic nude mice.

It may play an oncogenic role implying its significance in cancer development and its cellular functions needs to be investigated. On the contrary, NIH 3T3 cells overexpressing JS-2 did not display cellular transformation suggesting its overexpression may not be tumor transforming.

4) Bioinformatics search on JS-1 and JS-2 transcripts and proteins reveal no homology to known functional genes and proteins. JS-1 is predicted to contain target sites for kinase phosphorylation and it could possibly be involved in signal transduction related events and be targeted by kinases implicated in intracellular signal transduction pathways that normally lead to controlled cell growth. Further studies are required to understand its functional roles in this aspect.

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Appendix

General information on JS-1 gene

Accession No.	NM_138809
Definition	Homo sapiens hypothetical protein (LOC134147), mRNA
Chromosomal location	5p15.2
Length	2156bp
Coding sequence	245-982bp
Predicted protein	245 amino acids
Function	Unknown

General information on JS-2 gene

Accession No.	NM_199133
Definition	Homo sapiens hypothetical protein LOC134145, mRNA
Chromosomal location	5p15.2
Length	1842bp
Coding sequence	8-709bp
Predicted protein	233 amino acids
Function	Unknown