IDENTIFICATION AND CHARACTERIZATION OF NOVEL ONCOGENES LOCATED IN A HOMOGENEOUSLY STAINING REGION (HSR) OF HUMAN ESOPHAGEAL SQUAMOUS CELL CARCINOMA (ESCC)

CHRISTINA D. LIU

M.Phil

The Hong Kong Polytechnic University

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The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Identification and Characterization of Novel Oncogenes Located in a Homogeneously Staining Region (HSR) of Human Esophageal Squamous Cell Carcinoma (ESCC)

Christina Di LIU

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

July 2012

CERTIFICATE OF ORIGINALITY

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

_____ (Signed)

Miss Christina Di LIU

ABSTRACT

Esophageal squamous cell carcinoma (ESCC) has been reported to be caused by multiple genomic factors, such as amplification and overexpression of oncogenes, which lead to the malignant transformation of normal cells. In this study, a homogeneously staining region (HSR) was identified from an ESCC cell line SLMT-1 and was microdissected for FISH analysis to determine its chromosomal origin. The HSR was confirmed to be originated from chromosome 17p13.3 region with amplification and inversion. Further FISH analysis using the 17p BACs and array CGH analysis for chromosome 17p on SLMT-1 revealed that the amplicon was located within a 900kb region. According to the genomic data available at Ensembl release 46 (http://www.ensembl.org), mutliplex RT-PCR analysis for gene overexpression was performed for the eight non-tumor suppressor genes located within the amplicon on 14 ESCC cell lines compared to the immortalized non-tumor esophageal epithelial cell line. Among the genes studied, *JC-1* showed the highest percentage of overexpression in 14 ESCC cell lines (78.6%; 11/14), and the translocase of inner mitochondrial membrane 22 (*Timm22*) showed the second highest overexpression percentage of 71.4% (10/14). Thus *Timm22* and *JC-1* were further investigated for their oncogenic properties. Overexpression of *Timm22* and *JC-1* in NIH-3T3 cells could enhance the proliferation rates; enhance the

anchorage dependent and independent growth and the migration rates of transfected NIH 3T3 cells. Most importantly, the overexpression of *Timm22* and *JC-1* could also induce the formation of subcutaneous sarcomas in athymic nude mice. Immunohistochemical staining was performed for *Timm22* in seven ESCC cell lines compared with the immortalized non-tumor esophageal epithelial cell lines. Three out of seven (42.9%) ESCC cell lines showed Timm22 protein overexpression. Further multiplex RT-PCR analysis on the primary ESCC patient samples revealed that 16 out of 29 samples (55.2%) showed overexpression of *Timm22,* which was most commonly detected in Stage IIa (8/16; 50%); Stage III (6/16; 37.5%) and moderately differentiated tumors (9/16; 56.3%), but only one moderately differentiated Stage IIa tumor showed *JC-1* overexpression. The present study is thus the first study to demonstrate the oncogenic properties of *Timm22* and *JC-1* in ESCC with overexpression and the overall results suggest that *Timm22* and *JC-1* may play important roles in the molecular pathogenesis of ESCC.

PUBLICATIONS DIRECTLY RELATED TO THIS THESIS

Journal article:

Liu CD, Chan D, Tsoi MYT, Chan SH, Tao Q, Guan XY, Hu Liang, Law SYK, Chan KW, Tsao GSW,Lung ML,Lam AKY, JCO Tang. Overexpression and oncogenic properties of translocase of inner mitochondrial membrane 22 (Timm22) in esophageal squamous cell carcinoma. (*Paper in manuscript*)

Conference paper:

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LIST OF ABBREVIATIONS

ADH2	alcohol dehydrogenase
ADP	adenosine diphosphate
ALDH2	aldehyde dehydrogenase 2
AJCC	American Joint Committee on Cancer
ANT2	adenine nucleotide translocator 2
Array-CGH	array- comparative genomic hybridization
ATCC	American Type Tissue Culture
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BE	Barrett's esophagus
bFGF	basic fibroblast growth factor
BMI	body mass index
CAMs	cell adhesion molecules
CDKs	cyclin-dependent kinases
cDNA	complementary deoxyribonucleic acid
CDS	coding DNA sequence
CO_2	carbon dioxide
CGH	comparative genomic hybridization
CT	computed tomography
CYPs	cytochromes P450
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferases
DOC2B	double C2-like domains, beta
EADC	esophageal adenocarcinoma
EEC	early esophageal cancer
EDTA	ethylenediaminetetraaectic acid; ethylenediaminetetraaectate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMR	endoscopic mucosa resection
ESCC	esophageal squamous cell carcinoma
ESD	endoscopic submucosal dissection
EtBr	ethidium bromide
EtOH	ethanol
EUS	endoscopic ultrasonography
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GI	gastrointestinal
GSTs	glutathione S-transferases
GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase enzyme
H&E	hematoxylin and eosin
HER2	human epidermal growth factor receptor 2
HOX	homeobox
HPV	human papillomavirus
HSR	homogeneously staining region
hTERT	human terlomerase reverse transcriptase
hTP-1	human telomerase-associated protein
IARC	International Agency for Research on Cancer
INK4	inhibitors of kinase 4
KCl	potassium chloride
KSFM	keratinocyte serum-free complete medium
LATS2	large tumor suppressor homolog 2
LES	lower esophageal sphincter
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MEMα	minimum essential medium α
miRNA	microRNA
miR	microRNA
$MgCl_2$	magnesium chloride
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium]
MuLV	moloney murine leukaemia virus
NCBI	National Center for Biotechnology Information
PAC	P1-derived artificial chromosome
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PCR	polymerasae chain reaction
PDT	photodynamic therapy
PI3K	phosphatidylinositol 3-kinase
pRb	retinoblastoma protein
Rb	retinoblastoma
RNA	ribonucleic acid
RT-PCR	reverse transcription- polymerase chain reaction
SEER	Surveillance Epidemiology and End Results
SSC	saline-sodium citrate
Timm22	translocase of inner mitochondrial membrane 22
TGF	transforming growth factor

TNF	tumor necrosis factor
TNM	tumor-node-metastases
TP 53	tumor protein p53
Tris-HCl	tris- hydrogen chloride
TSGs	tumor suppressor genes
UES	Upper esophageal sphincter
UGI	upper-gastrointestinal
UICC	International Union Against Cancer
US/USA	United States of American
WHO	World Health Organization

LIST OF UNIT ABBREVIATIONS

µg	microgram
µl	micolitre
µM	micromolar
bp	base pair
cm	centimeter
cm^2	square centimeter
ng	nanogram
g	gram
	gravitational force
hr	hour
kp	kilo base pair
M	molar
Mb	megabase
mins	minutes
ml	millilitre
nm	nanometer
r.p.m	revolutions per minute
sec	second
^oC	degree Celsius

Chapter 1
Introduction and literature review

1.1. The esophagus

1.1.1. General features and anatomy of the esophagus

In human, the esophagus is a muscular passageway through which solids and liquids pass from the pharynx to the stomach after swallowing. It connects the mouth to the stomach and can be divided into cervical, thoracic, and abdominal components. The average length of the esophagus in adult human is around 25-30cm. The structures of the esophagus and the digestive system are shown in Figure 1. For the purpose of classification, staging, and reporting of esophageal malignancy, the International Union Against Cancer (UICC) has suggested division of the esophagus into four segments, with distances measured from the incisors. The cervical esophagus extends from the cricoid cartilage (roughly 15 cm) to the level of the thoracic inlet (approximately 18 cm). The upper thoracic segment extends from the thoracic inlet to the tracheal bifurcation (around 24 cm). The mid-thoracic segment extends to the level of the eighth thoracic vertebra (approximately 32 cm), and the lower thoracic segment extends to the junction with the stomach (40 cm) (Figure 2). The structure of the

opening of cervical esophagus from the pharynx is named upper esophageal (or inferior pharyngeal) sphincter. The sphincter is composed of the cricopharyngeus and inferior pharyngeal constrictor muscles. The lower part of the esophagus, namely lower esophageal sphincter (LES), is a muscular junction between the esophagus and the stomach. Peristalsis will occur subsequent to pharyngeal contraction and the opening of the upper esophageal sphincter which causes solids and liquids move through the esophagus (McFarland 2009).

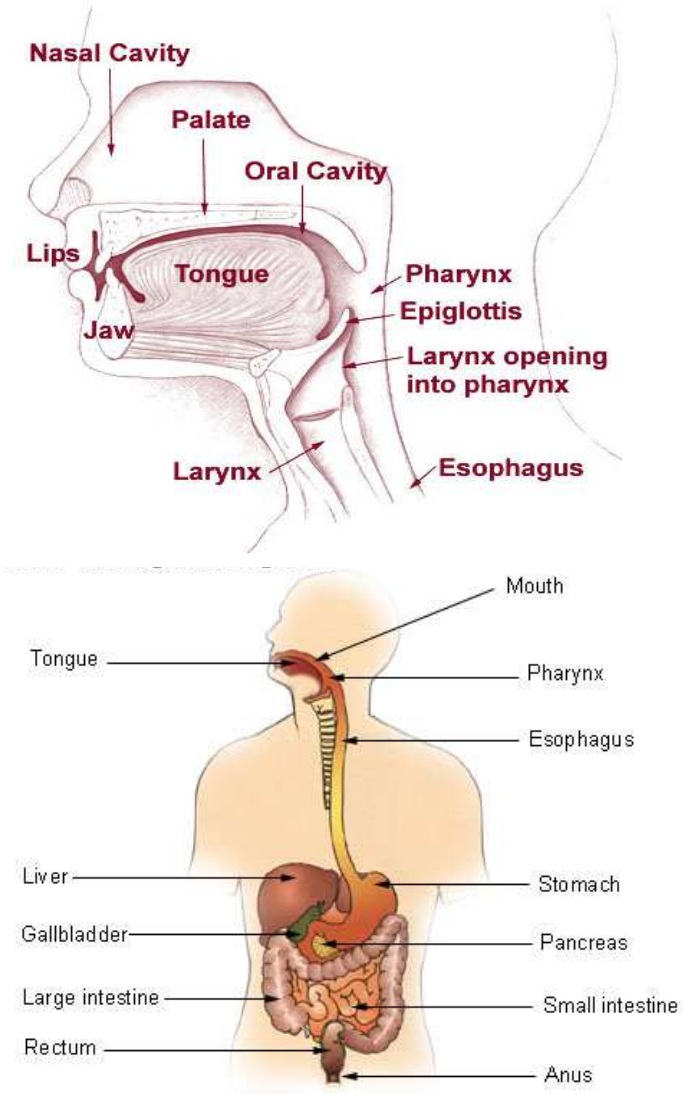


Figure 1. Structures of digestive system (upper) showing the position of the esophagus (upper and lower).

Sources:

http://training.seer.cancer.gov/head-neck/anatomy/overview.html

http://www.daviddarling.info/encyclopedia/D/digestive_system.html

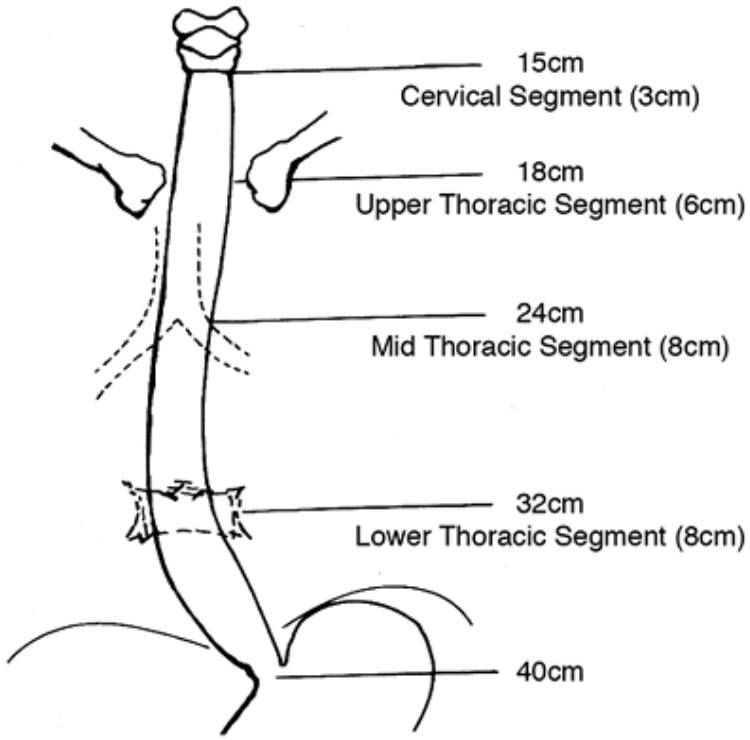


Figure 2. Esophageal segments with approximate lengths and distances from the incisors.
Adopted from (Mills and Sternberg 2006).

1.1.2 Histology of the esophagus

There are four main parts in the esophagus: mucosa; submucosa; muscularis externa and adventitia (Figure 3). The esophageal mucosa consists of three layers: epithelium, lamina propria and muscularis mucosae. The stratified squamous non-keratinized epithelium layer lines the esophageal lumen and is usually 0.5mm thick. The fibroelastic lamina propria is unremarkable and houses esophageal cardiac glands. The smooth muscularis mucosa comprises a single layer of longitudinally oriented smooth muscle fibers (Gartner and Hiatt 2001). The esophageal submucosa consists of a dense, fibroelastic connective tissue which houses the esophageal glands proper. The esophagus is a region of the alimentary canal with glands in the submucosa (Horvath and Kalmar 2009). The secretory units in the tubuloacinar glands are mucous cells and serous cells. There are apical accumulations of mucus-filled secretory granules in mucous cells which have basally located and flattened nuclei. Serous cells have round, centrally placed nuclei. Both types of cells contain the proenzyme pepsinogen and antibacterial agent lysozyme in their secretory granules. The secretions of these glands will go into the esophageal lumen by the ducts (Gartner and Hiatt 2001). The muscularis externa of the esophagus can be subdivided into two layers: inner circular and outer longitudinal. Finally, the esophagus is coated by adventitia until it pierces the diaphragm (Gartner

and Hiatt 2001).

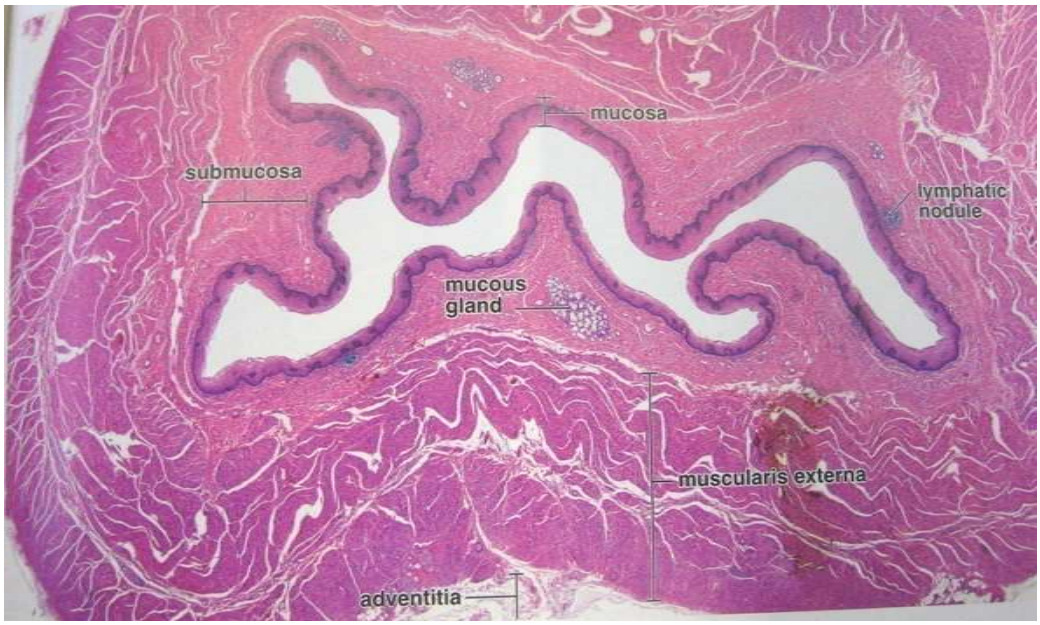


Figure 3. Photomicrograph of the hematoxylin and eosin (H&E)-stained cross-section of human esophagus (x100).
The four parts of the esophagus, mucosa; submucosa; muscularis externa and adventitia, are shown. (Ross and Pawlina 2006)

1.2. Esophageal Cancer

Cancer, whose medical term is malignant neoplasm, is a class of diseases in which a group of cells show uncontrolled growth, invasion and sometimes metastasis (National Cancer Institute – what is cancer http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer). All cancers result from abnormalities in genetic materials. Carcinogens could be one cause for the abnormalities and they may include radiation, tobacco smoke, chemicals, infectious agents and alcohol (Pavanello and Lotti 2012). A randomly acquired genetic abnormality through the normal DNA replication process could be present in all cells from birth and might eventually lead to cancer. Heritability of cancer is possibly a complex interaction between carcinogens and the genome of the host. Other aspects in cancer genetic pathogenesis such as DNA methylation and microRNAs are increasingly accepted as important (Li et al 2012). Esophageal cancer is the malignant growth of the esophageal tissues. Like most of the other cancers, esophageal cancer is a multi-factorial disease and the processes of carcinogenesis involve multi-stage events as seen in other cancers. These features will be discussed in the following sections.

1.2.1 Types of esophageal cancer

There are two more commonly found histological types of esophageal

cancer, namely esophageal squamous cell carcinoma (ESCC) which arises from the epithelial cells that line the lumen of the esophagus, and esophageal adenocarcinoma (EADC) which arises from glandular cells that are commonly found at the junction of the esophagus and stomach (Jobe et al 2009). ESCC comprises of an overwhelming majority of esophageal cancer and accounts for 90% of the cases in Hong Kong (Lam 2000). The histological subtypes of ESCC according to the levels of differentiation include well-differentiated, moderately differentiated and poorly differentiated types (Figure 4). ESCC can be found anywhere in the esophagus but most commonly happens in the upper two-third of the organ (Metzger et al 2004). EADC is the second most common type of esophageal cancer. It is mostly located in the distal esophagus (Lenhard et al 2001). There are some other types of esophageal cancer that are not as common as ESCC and EADC and they account for only 1-2% of all cancer cases. They include small-cell, primary malignant lymphoma, leiomyosarcoma, and neuroendocrine carcinoma (Lenhard et al 2001).

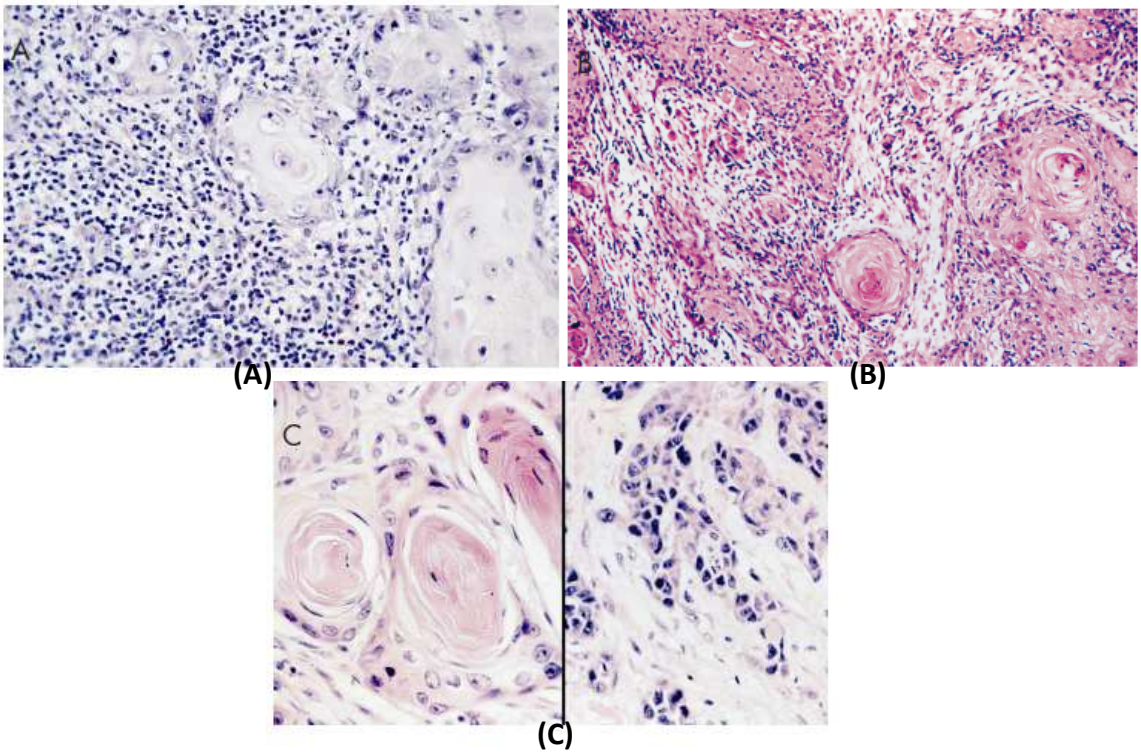


Figure 4. Histologic grades of ESCC.
A: Moderately differentiated. B: Well differentiated with prominent lymphoid infiltrate. C: Well differentiated areas (left) contrast with immature basal-type cells of a poorly differentiated carcinoma (right) (Adopted from WHO world cancer report 2008.
http://www.iarc.fr/en/publications/pdfs-online/wcr/2008/)

1.2.2 Tumor Staging

The stage of a tumor describes how much the cancer has spread. The stage often refers to the size of a tumor, how deep it has pierced, if the tumor metastasis occurred, how may lymph nodes has been affected, if it has invaded adjacent organs, and whether the distant organs are spread (Cancer staging at Wikipedia http://en.wikipedia.org/wiki/Cancer_staging). Most of the time, staging of the cancer is the basis for the choice of treatments. Tumor staging also predicts ESCC patients survival (Wick 2008). The American Joint Committee for Cancer (AJCC) provided the guidelines for the staging system of esophageal cancer in 1998. The divisions of the esophagus used in this staging system are cervical (up to 18 cm from the incisors), upper thoracic (18 – 24 cm), mid-thoracic (24 – 32 cm), and lower thoracic (32 cm, including the gastroesophageal junction). Surgical resection allows assessment of the pathologic TNM (tumor-node-metastases) stages. This staging system is adopted by both AJCC (American Joint Committee for Cancer) and UICC (Union Internationale Contre le Cancer). The TNM staging system is described in Table 1 and Table 2 illustrates the stage grouping for esophageal cancer.

Table 1. TNM Staging for Esophageal Cancer (Lenhard et al 2001)

Classification	**Definition**
Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor invades lamina propria or submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades adventitia
T4	Tumor invades adjacent structures
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
Tumors of the lower thoracic esophagus	
M1a	Metastasis in celiac lymph nodes
M1b	Other distant metastasis
Tumors of the midthoracic esophagus	
M1a	Not applicable
M1b	Nonregional lymph nodes and/or other distant metastasis
Tumors of the upper thoracic esophagus	
M1a	Metastasis in cervical nodes
M1b	Other distant metastasis

Table 2. AJCC/UICC stage Grouping for Esophageal Cancer (Lenhard et al 2001)

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T2	N0	M0
	T3	N0	M0
Stage IIB	T1	N1	M0
	T2	N1	M0
Stage III	T3	N1	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

1.2.3 Epidemiology

Esophageal cancer is an aggressive malignancy with poor prognosis. The mortality rate of esophageal cancer is also very high worldwide (Parkin et al 2005). In Hong Kong, esophageal cancer was the 8th most common cause of cancer death for both sexes and the 7th for male according to the statistics of leading cancer sites in 2009 available from Hong Kong Cancer Registry, Hong Kong Hospital Authority. Its mortality and geographic variability in incidence are higher than most other cancers (Boice et al 2006). The highest risk areas comprise the so-called Asian esophageal cancer belt stretching from eastern Turkey, via the southern former Soviet Union into the western and northern China, areas that include Kazakhstan, Turkmenistan, Tadjikistan and Uzbekistan, Iraq, Iran , Hong Kong, Japan, southeast Africa, France and parts of South America such as Brazil and Bermuda (Lam 2000). In addition, the white population in the United States and some western countries, an increased incidence of 350%, most of them are EADC, has been observed (Stathopoulos and Tsiaras 2003). Thus EADC surpassed ESCC and became the predominant tumor subtype while the ESCC incidence has not been changed within the same regions (Dreilich et al 2004, Holmes and Vaughan 2007). In Asia, including Hong Kong, the diagnosed esophageal cancers are still predominantly ESCC (Law and Wong 2004). All these evidences showed that the incidence and mortality of different

histologic subtypes of esophageal cancer varied considerably (Lam 2000).

1.2.3.1 Incidence, Mortality and Geographic Distribution

According to the World Cancer Report (WHO, 2008), developing countries have the highest incidence of esophageal carcinoma worldwide. Worldwide, the incidence of esophageal cancer ranked 6th among men and 9th among women in 2008. An estimate of 482,300 new esophageal cancer cases and 406,800 deaths related to esophageal cancer occurred in 2008 worldwide (Jemal et al 2011). The incidence of esophageal cancer ranks 6th among males worldwide. Females' top 10 incidences of cancer sites however do not include esophageal cancer. The mortality rate of esophageal cancer is higher which ranks 5th among males and 8th among females.(Jemal et al 2011) The 5-year survival rate of esophageal cancer is uniformly low and is usually less than 10% worldwide (WHO 2008). Although the 5-year survival rates of esophageal cancer have increased from 3.9% among men and 7.5 % among women in the 1970s to 18.4% among men and 18.8 % among women in the 2000s in the US (Figure 5), esophageal cancer remains one of the most deadly cancers and the prognosis is still rather poor (Medinfographics http://www.medinfographics.com/cancer-statistics/esophageal-cancer/esophageal-cancer-survival-by-year-of-diagnosis/).

The geographic distribution of esophageal cancer is extremely uneven. Figure 6 shows the age-standardized incidence rates of esophageal cancer. It can be seen that among women in the high-risk China and southern Africa their incidence rates of esophageal cancer are approximately 20 times higher than those in southern Europe. While in men, the difference is about 15-fold between high and low risk regions. It is commonly found that esophageal cancer is 3 to 4 times more common among males than females in most regions (Jemal et al 2011).

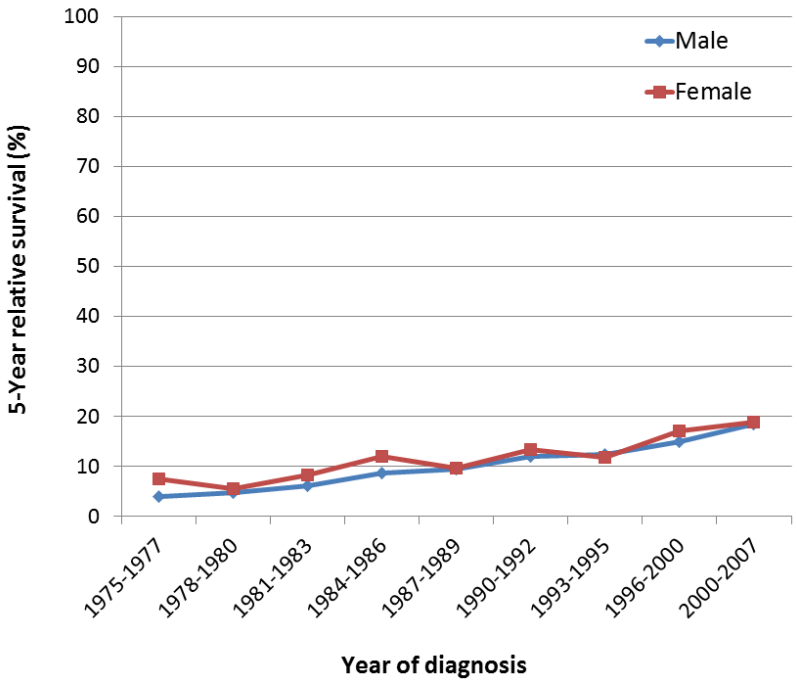


Figure 5. Esophageal cancer survival by year of diagnosis.

This figure shows the 5-year survival rates for esophageal cancer by year of diagnosis for the US male and female between 1975 and 2007 (Adopted from Medinfographics http://www.medinfographics.com/cancer-statistics/esophageal-cancer/esophageal-cancer-survival-by-year-of-diagnosis/ based on the most current survival data available for the US from the US National Cancer Institute's Surveillance Epidemiology and End Results (SEER) Data base).

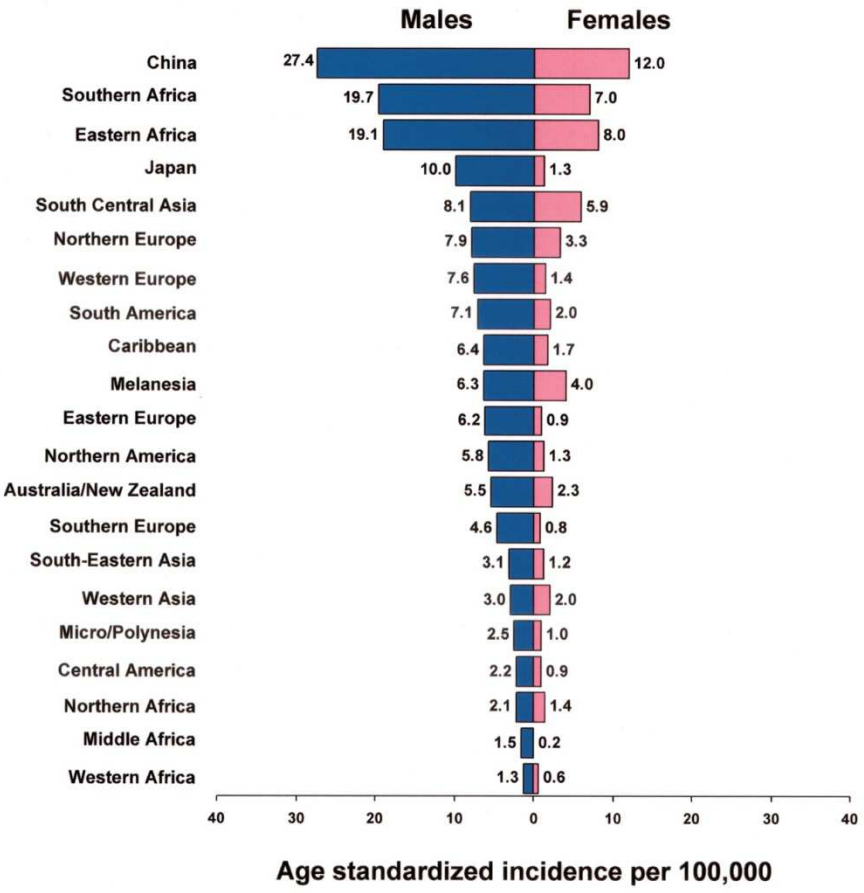


Figure 6. *Age-standardized* incidence rates of esophageal cancer.

(Parkin et al 2005)

1.2.3.2 Risk Factors

There are considerable geographic and racial variations in the incidence of this cancer, which are mostly explained by varying exposure to risk factors, although genetic susceptibility may play a partial role too. Many of the causative and risk factors for esophageal adenocarcinoma (EADC) and esophageal squamous cell carcinoma (ESCC) have been well discussed (Enzinger and Mayer 2003).

Tobacco and Alcohol

The positive correlation between heavy alcohol intake, tobacco smoking, and the risk of esophageal cancer has been well established (Toh et al 2010). The results for correlation of both smoking and alcohol habits with ESCC are consistently strong. The dose-risk trend with alcohol consumption has been described as linear, or more than linear (Adami et al 2008). Most authors found that no importance exists for types of beverage associated with the risk but wine might be protective compared to hard liquor (Pelucchi et al 2008). The effect of smoking is stronger for ESCC than EADC, with a recent cohort study showing that current smokers have a nine-fold risk increase for ESCC and a four-fold risk increase for EADC (Freedman et al 2007).

Diet and Nutrition

Dietary peculiarities have become the study focus as risk factors in high-incidence areas in Asia since tobacco and alcohol use appears to be small in Asia (Lucenteforte et al 2009). Many previous studies have established the correlation between alcohol consumption and cancer risk which also including esophageal cancer (Islami et al 2010, Pelucchi et al 2008, Pelucchi et al 2011, Seitz and Poschl 1997). It was suggested that consumption of fruit and vegetables is typically low in areas with the highest incidence of esophageal cancer (Hensrud and Heimburger 1998). Another analysis of food samples in high-incidence areas in Iran revealed that no remarkably high levels of aflatoxin, polycyclic aromatic hydrocarbons (PAH), or nitrosamines were found (Ghavamzadeh et al 2001). In addition, deficiencies in vitamin or microelement are documented in high-risk regions like Asia and South Africa (Adami et al 2008, Dlamini and Bhoola 2005, Lucenteforte et al 2009, Park et al 2008, Walker et al 2002).

An analysis of five case-control studies in the high risk areas of South America (northeastern Argentina, southern Brazil, Uruguay and Paraguay) confirmed that the consumption of very hot drinks was associated with a 2- to 4-fold increased risk of ESCC (Castellsague et al 2000). In this same area, the drinking of a locally grown herbal infusion, maté, was also positively

associated with esophageal cancer. The effect of maté would be increased if drunk at high temperatures. (Castellsague et al 2000, Sewram et al 2003)

Body Weight

Research work in Europe and South America has shown that obesity does not increase the risk of ESCC. In fact, a reduction in the risk of ESCC was shown with higher body mass index (BMI). (Castellsague et al 2000, Steffen et al 2009)

Infections

Infection with human papillomavirus (HPV) has been implicated as a risk factor for ESCC. It has been reported that HPV 16 and 18 were especially involved in the transformation of normal epithelial cells into tumor cells (Syrjanen 2002, zur Hausen 1999) especially in Africa (Hendricks and Parker 2002). However, HPV 16, 18 and 73 have been reported to have negative correlation with high risk ESCC area in China (Adami et al 2008). The explanation for the inconsistent results remains conjectural.

Occupation

Some occupations seem to be associated with increased risk for ESCC. Evidences have shown that individuals who work at vulcanization in the

rubber or automobile industry may suffer from higher risks of ESCC (Bouchardy et al 2002). Other occupations like chimney sweeps, mine workers, chemical products workers, butchers, and medical x-ray workers may also have increased risks of ESCC. In addition, workers in the cement industry, plastics and composites industry, the dye production industry, and bookbindery are also found to show significant positive associations (Adami et al 2008).

1.2.4 Molecular Aspects of ESCC

The molecular mechanism of esophageal cancer is still not fully understood. Many investigations have been conducted to understand more about esophageal carcinogenesis. In general, carcinogenesis is a multi-step process that abnormal metabolic and molecular changes were involved, and they lead to an excessive and untimely proliferation of cells. The following sections will discuss the topics related to the carcinogenesis of ESCC, namely etiopathogenesis, tumor-related genes, proliferation- related factors, factors related to metastasis, microRNA and oncogenes (Lam 2000).

1.2.4.1 Etiopathogenesis

Several recent research works on ESCC have shown the significance of viral etiopathogenesis and genes susceptible to chemical carcinogens, and they are believed to play a critical role in the molecular pathogenesis in ESCC.

1.2.4.1.1 Viral Etiopathogenesis

The relationship of human papilloma virus (HPV) and ESCC is conflicting in different geographic regions as indicated by the studies so far. High frequency of detecting HPV in ESCC was found only in France and Portugal among European countries and Japan, China, Hong Kong, India, Pakistan and Korea in Asia. ESCC in South Africa, Alaska and Australia also revealed HPV infection. HPV is a member of the papovavirus family with 73 different genotypes that can infect human skin and mucous membranes. Closed circular double-stranded DNA is found in this viral family (Hausen 1977). HPV-16 and -18 are the predominant types detected in ESCC (Turner et al 1997). It has been shown that HPV-18 E6 and E7 oncoprotein production can transform an embryonic esophageal epithelial cell line into a malignant cell type (Shen et al 2001). The study revealed that chromosomal aberrations, telomeric shortening and telomerase activity, and expression of certain genes are induced by the infection (Shen et al 2001). HPV E6 and E7 proteins

are able to activate telomerase activity and also activate oncogenes *c-myc* and *ras*. Tumor suppressor gene *p53* and *Rb* expression are also altered by these proteins, leading to the loss of their functions of tumor suppression, loss of cell growth control; apoptosis occurs and cellular transformation is resulted (Zhang et al 2007).

1.2.4.1.2 Chemical Susceptible Genes

Chemical compounds, synthetic or natural in environment may induce the development of human cancers. The uneven geographical distribution of ESCC cases may be related to the exposure of various toxic chemicals since different toxic chemicals are found in different regions. It is widely accepted that tobacco and alcohol consumption is epidemiologically associated with esophageal squamous cell carcinoma (Pelucchi et al 2008). Human susceptibility to cancer is shown to be affected by genetic polymorphisms in enzymes involved in metabolism of carcinogens. These genetic polymorphisms play a critical role in normal cell house-keeping activities which detoxify toxic compounds in the body (Lam 2000). A previous investigation has indicated that cytochromes P450 (CYPs) and glutathione S-transferases (GSTs) are enzymes produced by their genes and are related to the risk of ESCC (Nimura et al 1997). However, the studies by Lin *et al.* and Morita *et al.* have indicated that the genetic polymorphisms of

CYPs (CYP1A1, CYP2E1) and GSTs (GSTM1, GSTT1, GSTP1) have no association with cancer susceptibility in ESCC (Lin 1997, Morita et al 1997). On the other hand, alcohol metabolizing enzymes ADH2 (alcohol dehydrogenases) and ALDH2 (aldehyde dehydrogenases) were found to be associated with increased risk of ESCC (Hori et al 1997). Hori *et al*'s study showed that the $ADH2^{1}/ADH2^{1}$ and $ALDH2^{1}/ALDH2^{2}$ genotypes were significantly higher in esophageal cancer patients than in healthy controls. The activity of ADH [beta] 1 subunit encoded by $ADH2^{1}$ is low for alcohol metabolism, indicating that the delay of alcohol metabolism should be related to esophageal carcinogenesis (Hori et al 1997). Polymorphism of ALDH2 is also reported to have significant correlation with the occurrence of esophageal cancer in alcoholics. The frequency of $ALDH2^{2}$ in alcoholics is much lower than that in healthy controls and the homozygous type of $ALDH2^{2}$ exhibits no ALDH2 activity. These suggest a greater risk for esophageal cancer might occur in $ALDH2^{1}/ALDH2^{2}$ genotypes than $ALDH2^{2}/ALDH2^{2}$ homozygote (Hori et al 1997). The delay of acetaldehyde metabolism might also be associated with esophageal carcinogenesis since the $ALDH2^{1}/ALDH2^{2}$ homozygote exhibits little $ALDH2^{2}$ activity (Yokoyama et al 2001).

1.2.4.2 Tumor Related Genes

The development of human esophageal cancer is a multistep, progressive process. Recent studies frequently investigated genes related to ESCC carcinogenesis and development. The most interested genes include oncogenes, tumor suppressor genes, and those for maintaining telomeres, regulating apoptosis, repairing DNA, determining cellular characteristics, stimulating angiogenesis, assisting metastasis and microRNA (Lam 2000). Some genetic changes are consistently detected in ESCC regardless of patient's origin or suspected etiological factors. These changes include transformation of a normal gene into an oncogene, malfunctions in tumor suppressor genes, impairment of DNA repair pathway, and disruption of the G1/S cell cycle checkpoint that leads to loss of cell cycle control (Kuwano et al 2005).

1.2.4.2.1 Oncogenes

Oncogenes are genes that mutation or over-expression of them may transform normal cells into tumor cells. There are many researches done on oncogenes that are closely related to ESCC and they will be reviewed in Section 1.3.

1.2.4.2.2 Tumor Suppressor Genes

Tumor suppressor genes, or antioncogenes, protect cells from moving to the path of cancer development. Usually, if these genes are mutated or altered in expression, cells can progress into cancer according to the loss or reduction of their original tumor suppressing functions. Several tumor suppressor genes have been intensively investigated in previous research works of ESCC. They include *retinoblastoma (Rb), p15, p16, p21* and *p53*. Mutation, deletion, under regulation, loss of heterozygosity (LOH) and methylation are the ways that could inactivate tumor suppressor genes and the inactivation of tumor suppressor genes plays a key role in the tumorigenesis of esophageal cancer (Kuwano et al 2005, Lam 2000). Many tumor suppressor genes have the functions in producing proteins that regulate the processes of cell cycle, and subsequently, cell proliferation and differentiation. The control of cell cycle is believed to be conducted by two major pathways, the p53 (p14-MDM2-p53-p21) (Hollstein et al 1991a) and the retinoblastoma protein (pRb) (p16-cyclin D1-pRb) (Boynton et al 1991, Xing et al 1999c) pathways. p16 locus inactivation, *cyclin D1* overexpression and pRb inactivation could lead to deactivation of the pRb pathway while deactivation of the p53 pathway could be due to *p53* mutation, *MDM2* overexpression and *p14* inactivation. The pRb pathway controls cell proliferation while the p53 pathway regulates cell cycle arrest and apoptosis

(Kuwano et al 2005).

Retinoblastoma (Rb)

The *Rb* gene encodes retinoblastoma protein (pRb) which is a phosphoprotein that regulates the G1 restriction point in cell cycle by interacting with cyclin-dependent kinases (CDKs) such as CDK4, oncoproteins, and transcription factors like E2Fs (Weinberg 1995). pRb forms a complex with E2Fs and then negatively regulates the transcription. When pRb is phosphorylated by cyclin/CDK complex, E2Fs will be released and activates transcription and cell division (Chetty and Chetty 1997). Though deletions, mutations or inactivation of the *Rb* by HPV infection are uncommon, LOH of the *Rb* locus may play an essential role in inactivating of *Rb* (Kim et al 1997). In addition, apoptotic pathways of p53 are regulated by pRb via p14/ARF (Ciavarra and Zacksenhaus 2011). Some studies have shown that there was association between *Rb* mutations and advanced stage, lymph node metastasis, and reduced survival of esophageal cancers (Lin and Beer 2004).

p15* and *p16

p15 (*INK4b*; *MTS2*; *CDKN2B*) and *p16* (*INK4a*; *MTS1*; *CDK4I*; *CDKN2*; *CDKN2A*) are enzymes that belong to the INK4 (inhibitors of kinase 4) family which binds and inhibits the cyclin D1/CDK4 and CDK6 complex. They prevent cells from entering into S-phase in the cell cycle and inhibit cellular

proliferation (Tanaka et al 1997). The signals of DNA damage or senescence will activate p16 and then inhibit the function of cyclin D1/CDK4 and CDK6 complex. That will cause pRb phosphorylation and rarely leads to p53-independent G1 arrest (Furukawa 2002). p16 could be inactivated by homozygous deletion, mutation and abnormal DNA methylation according to the previous reports (Jobe et al 2009, Koh et al 2011, Nakakuki et al 2002, Rao et al 1995, Xing et al 1999b). Silencing of *p16* expression by methylation or LOH has been indicated in the early stage of carcinogenesis of ESCC, while later tumor progression involves the homozygous deletion of *p16* locus (Wong et al 1997). pRb will be inactivated if loss of *p16* expression and cyclin D1 overexpression occurs and this may result in poor prognosis of cancer (Li et al 2006). Unlike *p16*, *p15* is stimulated by transforming growth factor-β (TGF-β) and activation of *p15* will thus activate G1 arrest (Thillainadesan et al 2008). Nearly half of ESCC patients showed *p15* alterations, with both homozygous deletions and mutations found (Xing et al 1999a). In summary, the simultaneous inactivation of *p15 and p16* genes results in the loss of the pRb-regulated restriction point and is crucially involved in esophageal carcinogenesis.

p21

p21 (*WAF1*; *CIP1*; *CAP20*; *PIC1*; *SDI1*; *CDK-interacting protein 1*; *CDKN1A*;

wildtype p53-activated fragment 1) encodes a cyclin-dependent kinase (CDK) inhibitor which is directly upregulated by a wild-type p53. G1 arrest after DNA damage is mediated by *p21* through the accumulation of hupophosphorylated pRb (Slebos et al 1994). Mutation and deletion of p21 in human cancers, including ESCC, is rare (Shiohara et al 1994). However, polymorphisms of *p21* have been identified at codons 31 and 149 in exon 2, and may play a role in the tumorigenesis of ESCC (Bahl et al 2000). Some researchers have found that reduced expression of *p21* demonstrates a poorer prognosis in ESCC (Natsugoe et al 2000, Nita et al 1999), while others have found no significant correlation (Masuda et al 2003, Shimada et al 1999). Therefore, the role of *p21* expression for prognosis in ESCC is still controversial. Several studies have indicated that wild type p53 concomitant with *p21* expression may give a positive outcome to chemotherapy or chemo-radiotherapy in ESCC (Michel et al 2002, Shimoyama et al 1998).

p53

p53 encodes a transcription factor that plays a critical role in maintaining cellular integrity and suppressing cellular transformation (Lin and Beer 2004). *p53* mutation is the most common genetic alteration in human cancers and the most frequently studied genetic alteration in ESCC. The role of p53 in cell

cycle is described in Figure 7. If there is DNA damage, product of *p53* stops the cell from going into S phase and this initiates cell cycle arrest via p21. Cells would be able to repair the damaged DNA before the *p53* inducing apoptosis if the DNA damages fail to repair (Kuwano et al 2005). Inactivation of the p53 pathway is caused by the mutation of p53 itself. Based on the information from the International Agency for Research on Cancer (IARC) tumor protein p53 (*TP53*) mutation database, more than 92% of the mutations are located in four evolutionary conserved domains of *p53* gene, namely exon 5 to exon 8, with hot spots at Arg175, Cys176, Arg248, Arg 273, and Arg282 (Olivier et al 2002). More than 80% of the mutations are point mutations, among which 46% are transition mutation and 36% transversion mutations (Kuwano et al 2005). Among these mutations, *p53* point mutations with G→T transversion are commonly found in ESCC (Bahl et al 2000). Chemical carcinogens from cigarette smoke, alcohol and alfatoxin may be responsible for this transversion and this explains the reason for tobacco, alcohol and fungal contaminated food being considered risk factors of ESCC (Kato et al 2001, Saeki et al 2000). Apart from that, geographical location is also a factor in the different patterns of *p53* mutations observed. For example, mutations of *p53* in exon 5 are commonly found in mainland China (Shao et al 2008). Mutations in exons 6 and 7 are frequently present in cases from Taiwan (Lee et al 2000), and

exon 7 (codons 248, 273) and 8 (codon 285) in ESCC patients in Hong Kong (Lam 2000). Among all *p53* mutations in ESCC, 68% of the point mutations results in missense mutation and 9.8% and 13% of them result in nonsense (stop codon) mutation and frameshift mutations (insertion and deletion) respectively (Egashira et al 2007). Mutation of *p53* is found to be linked with early stages of ESCC and may contribute to tumor progression as well as resistance to multi-modal treatments (Kitamura et al 1996, Parenti et al 1995). Some studies have suggested that the presence of mutant type *p53* is correlated to a poor prognosis in ESCC while some others have demonstrated that it correlated with prognosis (Lin et al 2009).

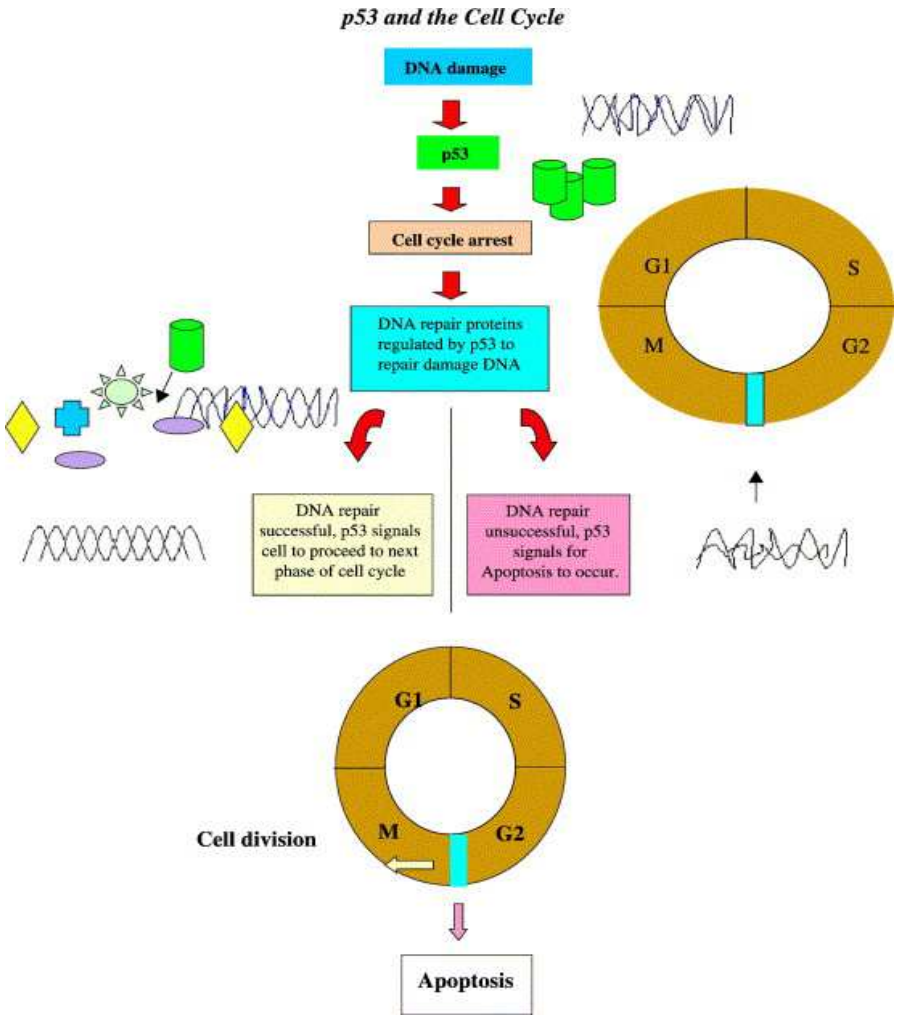


Figure 7. Role of p53 in cell cycle: mediator between cell growth and death. (i) During each checkpoint of the cell cycle, the cell cycle is arrested for p53 (and other tumour suppressor genes) to check if DNA damage has occurred. If DNA damage has occurred, p53 would stop the cell cycle at the checkpoint and direct appropriate proteins to repair the damage. Once the damage has been repaired, p53 would signal cell to continue the cell cycle into the next phase. (ii) If DNA damage is too severe to repair, p53 would signal apoptotic inducing factors (AIF), like the Bcl-2 family, to induce apoptosis rather than proliferation that would cause damage to the organism. (McCabe and Dlamini 2005)

1.2.4.2.3 Metastasis

Metastasis is a complex series of steps through which cancer cells migrate to other parts of the body through the bloodstream or lymphatic system. Cell adhesion molecules (CAMs) and the enzymes in degradation of extracellular matrix are factors that relate to metastasis (Lam 2000). Cell adhesion molecules are in charge of regulating cell-cell and cell-matrix interactions (Katz et al 1991, Streit et al 1996). Moreover, cell adhesion molecules play a role in metastasis of malignant tumors to the body tissue by combining with tumor cells (Lam 2000). Cadherins are calcium-dependent cell-cell adhesion transmembrane glycoproteins anchored to the cytoskeleton via cytoplasmic proteins such as alpha and beta catenin. E-cadherin, one of the classical cadherins, is mostly reported cell adhesion molecule included in implicating metastasis of ESCC (Bracke et al 1996). Cell-cell adhesion is implicated by E-cadherin by associating with a cytoplasmic complex of catenins linked to the actin cytoskeleton (Smith and Pignatelli 1997). Demosomal glycoprotein, a major adhesive component of the desmosome, is another subtype of CAMs. Reduction of E-cadherin, desmosomal glycoprotein and catenin expression is correlated to tumor dedifferentiation, infiltrative growth, lymph nodes metastases, hematogenous recurrence and poor prognosis in ESCC since it decreases cell adhesion, facilitates tissue invasion and consequently metastasis (Jian et al 1997, Kadowaki et al 1994, Kimura et al

1999, Nakanishi et al 1997, Natsugoe et al 1997, Natsugoe et al 1998, Sanders et al 1998, Takayama et al 1996, Tamura et al 1996). Moreover, enzymes that are involved in the degradation of extra-cellular matrix are also important for enhancing tumor cell movement. Matrix metalloproteinase (MMPs), plasminogen activator and cathespins are examples for this kind of enzymes (Shima et al 1993). Particularly, MMPs, a family of nine or more highly homologous zinc enzymes (endopeptidases), is commonly investigated among the extra-cellular matrix degradation related enzymes. In ESCC, the expression of MMPs-1, 2, 3, 7 and 9 has been studied by immunohistochemistry and more than half of ESCC cases have shown expression of MMPs (Adachi et al 1998, Murray et al 1998, Porte et al 1998, Shima et al 1992). *nm23* is another important gene involved in metastasis suppression (Backer et al 1993). Patel *et al.* have shown that the product of *nm23* could probably regulate cell functions using microtubules that are involved in mitotic spindle formation and cell locomotion. Cell signal response may also be regulated by *nm23* product through physical association with G proteins. Loss in expression of *nm23* are found in 63% of ESCC patients (Patel et al 1997).

1.2.4.2.4 Apoptosis genes

Apoptosis is a process of genetically programmed cell death that

naturally eliminates cells in response to particular stimuli (Gerl and Vaux 2005). The *bcl-2* family of proteins is believed to be the most important regulators of apoptosis. Genes involved in this family produce both anti-apoptotic proteins such as *bcl-2* and *bcl-X_L* and pro-apoptotic molecules like *bax* and *bak*. Different studies have shown that *bcl-2* gene is essential for regulating apoptosis in ESCC with 32% to 74% overexpression (Koide et al 1997, Ohbu et al 1995, Ohbu et al 1997, Parenti et al 1997, Puglisi et al 1996, Sarbia et al 1996, Sarbia et al 1998). The increased expression levels of the *bcl-2* family of proteins found in ESCC also include *bax* and *bcl-X_L* (Sarbia et al 1997) (Torzewski et al 1998). Cell growth is limited and homeostasis is maintained by apoptosis. Overall, any imbalance of cell proliferation and/or cell death due to of cell cycle and apoptotic regulators alterations can contribute in neoplasms growing.

1.2.4.2.5 Telomere related genes

Repetitive telomere sequences are found at the ends of eukaryotic chromosomes to protect the ends from damage and rearrangement. Cell division and DNA replication are regulated by progressive shortening of telomeric sequences (Kyo et al 2000). More than 80% of ESCC cases as well as their preneoplasia and lesions are found to have detectable telomerase activity (Seimiya et al 2002). In addition to telomerase activity, mRNA

expression of human telomerase reverse transcriptase (hTERT) and human telomerase-associated protein (hTP-1) are also overexpressed in ESCC (Li et al 2003). In contrast, telomerase activity is not detectable in most normal tissues and somatic cells (Harley et al 1990). In normal tissues, telomere will shorten with subsequent rounds of cell division, and the shortening could result in eventual loss of vital genetic information, which leads to reduced cellular proliferative capacity and cell death (Huard and Autexier 2004). Most types of human cancers are detected with telomerase activity, suggesting telomerase could be one of the most common tumor markers (Shay and Bacchetti 1997). Among the cancers with telomerase activity detected, ESCC is also found to have telomerase activity (Seimiya et al 2005). The activation of telomerase can demonstrate an infinite replicative capacity and maintain the length of telomeres so that cancer cells will bypass telomere crisis and therefore results in tumorigenesis (Seimiya et al 2002). A previous study by Morales *et al* indicated that reactivation of telomerase could be a major mechanism for immortalization of most human cancers cells, and according to the telomerase activity, it has become a diagnostic and prognostic marker for malignant tumors (Morales et al 2003). Some previous studies have also suggested that by targeting telomerase, there might be an advanced therapy against ESCC that could specifically aim at telomerase-positive tumor cells while most of human somatic tissues are

telomerase-negative (Seimiya et al 2002).

1.2.4.3 Loss of heterozygosity (LOH)

Loss of heterozygosity (LOH) refers to the mutation that leads to the loss of normal function of an allele of a gene when the other allele is already inactivated. When an activating mutation in one allele of a tumor suppressor gene is passed on to its offspring, LOH will occur if the remaining functional allele that contains a tumor suppressor gene is inactivated by mutation. Consequences of LOH include the dysfunctioning of tumor suppressor or even the ceasing of its production, both of which would lead to carcinogenesis. Potential LOH regions in the genome that contain putative tumor suppressor genes could be identified by microsatellite markers and LOH studies. Some researchers have suggested that allelic losses at chromosomes 1p, 3p, 4p, 4q, 5q, 9p, 9q, 11q, 13q, 17p, 17q and 18q are commonly found and may relate to ESCC development (Holland and Bast 2000) and (Stoner and Gupta 2001). Specific candidate genes implicated in the early stage of carcinogenesis as revealed by LOH study include *p16* gene on chromosome 9p21. The LOH on chromosome9p21 is a common mechanism for *p16* inactivation (Cai et al 2007). LOH at chromosome 5q31 may be associated to etiopathogenesis of ESCC (Mandard et al 2000). Moreover, inactivation of Rb and p53 in human ESCC might

also be affected by LOH of Rb and p53 loci on chromosome 13q14 and 17p13 (Mathew et al 2001). Apart from that, LOH was also found on chromosome 17q25 and related with tylosis, which is a rare autosomal dominant syndrome associated with high predisposition to ESCC (Mandard et al 2000).

1.2.4.4 Methylation

Methylation mainly refers to the process through which a hydrogen atom is replaced by a methyl group. If methylation happens at the promoter region of a given gene, the gene may be transcriptionally silenced by the process. Thus the tumor suppressor genes in many types of human cancers may be inactivated due to the loss of protein expression because of methylation (Roncalli et al 1998). The importance of methylation also relates to ESCC. The mechanism of DNA methylation consists of the action of DNA methyltransferases enzymes (DNMT 1, 3a or 3b), which leads to the production of 5-methylcytosine (Cheng and Blumenthal 2008). Several studies have shown that the Wnt signaling pathway or the p53 network is involved in the methylation of genes that are related to ESCC pathogenesis. The CpG islands (genome regions with high levels of cytosine-guanine repeats) are methylated at the promoter regions and this will lead to downregulation of E-cadherin that facilitates tissue invasion and metastasis

in human ESCC (Yoshiura et al 1995). In addition, putative tumor suppressor gene included in *p53* network inactivation is another example associated with methylation. Hypermethylation of *p16* gene in the *p53* network is frequently shown in ESCC cases in Linxian, China (Xing et al 1999b).

1.2.4.5 MicroRNAs

MicroRNAs (miRNAs) are involved in the biological and pathologic processes of cancer, including cell differentiation, proliferation, apoptosis and metabolism, and are emerging as highly tissue-specific biomarkers with potential clinical applicability for defining cancer types and origins (Rosenfeld et al 2008). Although miRNAs can function as oncogenes or tumor suppressors (Croce 2009), the functional information of miRNAs in ESCC is still not fully investigated. Recently, many researches have reported the miRNA expression pattern in ESCC. In general, research by Feber A et al. showed that miR-21 expression in both squamous cell carcinoma and adenocarcinomas was 3 to 5 times higher than in normal epithelium. Besides, expression of miR-203 and -205 in both tumors was 2 to 10 times less than in normal epithelium (Feber et al 2008). The miR-21 expression was also elevated as reported in another study based on a microarray result in 70 ESCC patients, and miR-375 showed reduced level in ESCC tissue compared with non-tumor tissue (Mathe et al 2009). In Ogawa et al.'s report,

the overexpression of miR-219 was identified as a significant and independent prognostic factor in surgically treated ESCC patients (Ogawa et al 2009). Large tumor suppressor homolog 2 (LATS2) was suggested to be suppressed by miR-373 so that miR-373 bears a potential oncogenic function (Lee et al 2009). Hiyoshi et al. also indicated the significant elevated expression of miR-21 in ESCC tissues and cell lines. They also found that miR-21 could regulate cell proliferation and invasion through suppressing an apoptosis-related molecule, programmed cell death protein 4 (Hiyoshi et al 2009). In addition, an multivariate analysis showed a correlation between elevated expression of miR-103 and -107and poor survival of ESCC patients (Guo et al 2008). Moreover, Matsushima K et al. conducted a microRNA microarray analysis with miRNAs extracted from ESCC cell lines which revealed significant alterations of miR-205 and -10a expression levels in cellular expression (Matsushima et al 2010). Functional studies of miR-205 showed that it is likely to control cell invasion and migration in ESCC cells, through its repression of ZEB2, as a repressor of E-cadherin. Similarly, a possible role of miR-10a may involve in suppressive mechanisms of metastatic behavior of the cancer cells as it targets homeobox (HOX) genes (Matsushima et al 2010).

According to the previous findings as summarized above, it is obvious that miRNA plays an important role in the diverse molecular mechanisms

that lead to the formation and progression of esophageal cancer, including both ESCC and EAC. It is believed that most miRNAs are associated with esophageal cancer and their molecular characteristics are involved in the progression of esophageal cancer. The importance of miRNA in esophageal cancer is recently noticed. More research work will be done to understand the correlation between miRNA and esophageal carcinogenesis (Li et al 2011).

1.3. Literature Review of Oncogenes in ESCC

Some previous studies have reported the significance of oncogenes that are involved in the pathogenesis of ESCC, which include growth factors, growth factor receptors, signal transducers, and nuclear factors. Mutation, amplification and/or overexpression of oncogenes are the major mechanisms for the carcinogenesis of ESCC (Lam 2000) and (Kuwano et al 2005).

1.3.1 Growth Factors

Growth factors stimulate cells to enter the cell cycle from the resting phase (G0). Epidermal growth factor (EGF) and transforming growth factor (TGF)-α and *c-sis* function as autocrine growth factors for ESCC *in vitro* (Jones et al 1993, Wong et al 1994, Yoshida et al 1990a). Amplification of *hst-1* (FGF3) and *int-2* (FGF4) is commonly found in ESCC and associated with aggressive behaviors (Kitagawa et al 1991, Tsuda et al 1989, Yoshida et al 1990b). *hst-1* and *int-2* genes encode the basic fibroblast growth factor (bFGF) and are found to be significantly correlated with a high incidence of eventual metastasis in distant organs in ESCC patients (Kitagawa et al 1991). However, the amplification may not be accompanied by overexpression of *hst-1* and *int-2* genes (Tsuda et al 1989). Moreover, amplification of a nuclear

regulatory protein *cyclin D1* gene was found in ESCC with association of amplification of *hst-1* and *int-2*. It was reported that all the genes with amplification may not be associated with protein overexpression except cyclin D1 (Chetty and Chetty 1997). Overall, besides studies in cell lines, there is insufficient clinical data about the role of growth factors in ESCC. There is no evidence that growth factors play any important role in ESCC (Lam 2000).

1.3.2 Growth Factor Receptors

Epidermal growth factor receptor (EGFR) protein family is located on cell membrane and acts as receptors which have intracellular tyrosine kinase activity and extracellular binding domain for signaling transduction. EGFR family consist of *erbB-1* (*HER1*; *EGFR*), *erbB-2* (*HER*), *erbB-3* and *erbB-4* (Burgess and Thumwood 1994, Issing et al 1993, Plowman et al 1993). The binding domain of EGFR could bind to specific ligands, such as epidermal growth factor (EGF) and transforming growth factor (TGF)-α, and thus could activate the intrinsic tyrosine kinase, which would lead to the initiation of the Ras-Raf-MAP-kinase pathway for cell survival (Salomon et al 2000). Phosphatidylinositol 3-kinase (PI3K) and the downstream protein kinase Akt constitute to another important pathway that regulates multiple biologic processes, such as apoptosis, gene expression, and cellular proliferation. The

regulation is signaled by activation of Akt in the pathway. Akt could launch antiapoptotic signals by phosphorylating multiple targets and lead to the regulatory function in cell cycle progression.(Blanco-Aparicio et al 2007). Overexpression of EGFR was found in both ESCC cell lines and patient tumor samples. The EGFR gene amplification may be one of the mechanisms of activation and the overexpression pattern of EGFR correlates with poor prognosis (Grugan et al 2010). In addition, overproduction of *erbB-2* (HER2) was also reported by some studies but it is less commonly found in ESCC than adenocarcinoma (Yokota et al 1986). This may indicate that the activation of *erbB-2* plays a role in the early stage of ESCC development although it does not act as a prognostic marker.

1.3.3 Signal Transducers

The *ras* gene family is structurally related to a 21kDa protein namely ras p21. p21 is a member of the G protein superfamily which acts as mitogenic signal transducer and regulates cell growth and differentiation by the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway (Stoner and Gupta 2001). In ESCC, the *ras* oncogene family is not mutated though mutation could be detected in many human cancers (Hollstein et al 1991b). However, ras p21 protein is overexpressed in ESCC and is more often found in cytoplasm of well and moderately differentiated ESCC (Lam et al 1995,

Ono et al 1994, Ruol et al 1990). It is suggested that overexpression of *ras* p21 might contribute to a higher proliferative rate of cancer cell (Lam 2000).

1.3.4 Nuclear Factors

Cyclins are proteins that form a complex with proper cyclin-dependent kinase (CDKs). Cyclin D1 is a protein encoded by *CCND1, PRAD1, or bcl-1* gene on chromosome 11q13. It is involved in the p16-pRb pathway to effectively control the cell cycle (Motokura et al 1991). By complexing cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6), cyclin D1 can induce phosphorylation of pRb, which would release transcription factors E2Fs and thus results in a transition from G1 to the S phase (Lew et al 1991). In S phase, cells undergo DNA synthesis and if overexpression of c*yclin D1* happens, the cell may be out of control and transformed into a malignant phenotype (Baldin et al 1993). Amplification of 11q13 may result in the overexpression of c*yclin D1*, which is commonly observed in several human cancers, such as ESCC (Gaudray et al 1992, Tsuruta et al 1993). *Cyclin D1* overexpression is believed to be involved in dysplasias and early cancer (Shamma et al 2000), as well as in enhancing cell transformation (Watanabe et al 1999). In addition, the amplification of cyclin D1 gene or its protein accumulation can predict a poor prognosis in ESCC, according not only to univariate analysis (Naitoh et al 1995, Takeuchi et al

1997), but also to multivariate analysis (Sarbia et al 1999, Shinozaki et al 1996). In summary, previous findings supported that c*yclin D1* is a critical parameter in predicting an unfavorable overall survival in ESCC patients (Lam 2000).

Chapter 2
Aims and objectives of the thesis

Although previous researchers have discovered some genetic alterations related to ESCC, the overall picture of the molecular pathogenesis of ESCC is still not fully understood. As mentioned in the introductory section, the investigation on the oncogenes is critical for the further understanding on the pathogenic mechanisms of cancers, including ESCC. This MPhil study aims to identify novel oncogenes that are involved in the molecular pathogenesis of ESCC. In this research, a Homogeneously Staining Region (HSR) located at chromosome 17p13.3 region was identified and characterized in an ESCC cell line SLMT-1, which is of Hong Kong Chinese origin. Further study of the novel candidate oncogenes located within the HSR was performed. Identification of novel ESCC-associated genes therefore may contribute to the understanding of the pathogenesis of the disease and may possibly suggest the potential therapeutic targets to improve the survival rate of ESCC patients in the future research. The present study has two aims:

(I) To characterize the newly identified homogeneously staining region (HSR) in SLMT-1;

(II) To study the oncogenic nature of the novel genes which are located within the HSR;

Based on the aims of the present study, the following objectives were derived:

(i) To characterize the chromosomal position and nature of the identified HSR;

(ii) To examine the expression level of the genes located within the HSR, namely *JC-1, NP3694, Fam101B, Fam57A, Q9NRE4, RNMTL1, Q6ZP06* and *Timm2*2 in non-tumor esophageal epithelial cells and ESCC cell lines;

(iii) To determine the expression status of *JC-1* and Timm22, which showed the highest frequency of overexpression in ESCC cell lines and in the surgical specimens of ESCC;

(iv) To correlate the *Timm22* and *JC-1* overxpression profiles with the clinicopathol*o*gical features of the ESCC samples.

(v) To evaluate *in vitro* and *in vivo* growth transforming properties of *Timm22* and *JC-1*

The results of investigating the above objectives may provide further understanding on the roles these newly identified oncogenes, *JC-1* and

Timm22, play in the molecular pathogensis of ESCC. They may provide ground work for further investigation of other human cancers.

Chapter 3
Materials and Methods

3.1. Tumor and Non-tumor Cell lines

3.1.1 ESCC cell lines

A total of 15 ESCC cell lines were studied in this project. Five of them are of Chinese origin (SLMT-1 (Tang et al 2001b), HKESC-1 (Hu et al 2000), HKESC-2, HKESC-3 (Hu et al 2002) and HKESC-4(Cheung et al 2007)) and the other 10 are of Japanese origin (KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE270, KYSE410, KYSE450, KYSE510 and KYSE520) (Shimada et al 1992) and all were purchased from DSMZ (Braunschweig, Germany). The KYSE series of the ESCC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) or DMEM/F12 (Invitrogen) medium containing 10% fetal bovine serum (FBS) and antibiotics; HKESC-1, HKESC-2, HKESC-3 and HKESC-4 were cultured using MEMα (minimum essential medium α) medium (Gibco) with 10% fetal bovine serum and antibiotics (100μg/ml penicillin and 100μg/ml streptomycin). All the cells were maintained at 37°C in a humidified incubator with 5% CO_2. Trypsinization was performed if the density of cells was over 80% and it was done by adding phosphate buffered saline (PBS) for washing followed by adding 0.05% trypsin in 0.05%

EDTA (Gibco) for generating cell pellets. A splitting ratio of 1:5 was used for subculturing. The cells pellets were kept at -80C for later DNA or RNA extraction.

3.1.2 Immortalized non-tumor esophageal epithelial cell lines

The immortalized non-tumor esophageal cell lines NE1 (Deng et al 2004) and NE3 (Zhang et al 2006) were examined in the present study as the controls and they were kindly provided by Professor George S.W. Tsao of the Department of Anatomy, The University of Hong Kong. The culture medium for NE1 and NE3 was keratinocyte serum-free complete medium (KSFM, Gibco) without any serum and with antibiotics (100μg/ml penicillin and 100μg/ml streptomycin), and were kept at 37°C in a humidified incubator with 5% CO_2. The steps of subculturing were the same as described in the previous section. The cells pellets were kept at -80C for later DNA or RNA extraction.

3.1.3 Non-tumor fibroblast cell line

NIH 3T3 cells, a mouse fibroblast cell line, were purchased from the American Type Culture Collection (ATCC) and used as the recipient cells for the transformation studies of novel oncogenes. The culture medium was

Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS, 100μg/ml penicillin and 100μg/ml streptomycin. The cells were maintained in a 37°C humidified incubator with 5% CO_2. The steps of subculturing were the same as described in the previous section. The cells pellets were kept at -80C for later DNA or RNA extraction.

3.2. ESCC patient specimens

Thirty-one archived ESCC paired patient specimens (non-tumor and tumor) were used for the study of *JC-1* and twenty-nine specimens were used for *Timm22* expression. The ESCC tumor specimens were collected from the Department of Surgery, Queen Mary Hospital, Hong Kong, during 1990-2001 after ESCC patients had undergone esophagectomy. Their corresponding non-tumor epithelial tissue specimens were collected for comparison and located at least 10cm away from the tumor. The histopathological features of the patients were collected with consent from the Department of Pathology, Queen Mary Hospital, and summarized in Table 5 and Table 6 in Chapter 4.

3.3 G-banding analysis

Eighty percent confluent cell cultures (using 25cm^2 culture flask) of SLMT-1 were treated with colcemid at a final concentration of 0.1μg/ml for 4 hours at 37°C. The SLMT-1 cells were harvested by trypsinisation as described in the previous section. The harvested cells were mixed with 8ml hypotonic solution of 0.075M KCl and incubated at 37°C for 12 minutes. The SLMT-1 cells were then fixed by adding 2ml fixative (3 parts of methanol to 1 part glacial acetic acid) and the cells were collected by centrifugation at 463g. The residue was finally resuspended into 2ml of the fixative. The metaphase chromosome spread was prepared by dropping a drop of the fixative onto the top of a glass slide. The G-bands of the metaphase chromosome spread were examined by pretreatment with trypsin followed by staining with Leishman's stain (Sigma). Briefly, the prepared slides were warmed at 95°C for 20 minutes. The slides were immersed in 0.025% trypsin in normal saline for 120 seconds. The trypsinisation was stopped by immersing the slides into phosphate buffer (pH 6.8). The slides were flooded by the solution containing 1 part of Leishmann's stain with 3 parts of Gurr buffer (Biomedical Specialties) for 2 minutes. The slides were then rinsed with distilled water and dried at 60°C for 1 hour before mounting. The karyotypes of each metaphase were observed under light microscope to identify the HSR.

3.4 Chromosome microdissection and FISH analysis

Chromosome microdissection, PCR amplification of microdissected DNA from the HSR region, and fluorescence *in situ* hybridization (FISH) analysis were performed as described previously (Hu et al 2004). In brief, metaphase spreads of SLMT-1 cell line were G banded and six copies of partial HSR region were dissected. The dissected DNA was then amplified by PCR with UN1 primer and T7 DNA polymerase (Sequenase Version 2.0, USB, Cleveland, OH) for 8 cycles. Then this amplified DNA was further amplified using Taq polymerase for 30 cycles. Amplified DNA was labeled with spectrum orange dUTP (Vysis, Downers Grove, IL) by PCR, and then hybridized to both SLMT-1 ("back FISH") and normal metaphase spreads as previously described (Hu et al 2004). To further determine the chromosomal nature of the 17p HSR, a 3-color FISH was performed on the SLMT-1 metaphase spread by using the three bacterial artificial chromosome (BAC) clones of 17p (RP11-1260E13, RP11-199F11 and RP11-809H20 kindly provided by Dr. Hu Liang, Department of Clinical Oncology, The University of Hong Kong. The BAC clones were selected based on the FISH results of probing the microdissected DNA from the HSR region on the metaphase spread of normal lymphocytes. Figure 8 shows the chromosomal positions of the three BAC clone used in this FISH

analysis. For the labeling of probes, the hybridization procedures were followed as previously described in this section. Chromosomal images were captured using fluorescent light microscope (Olympus).

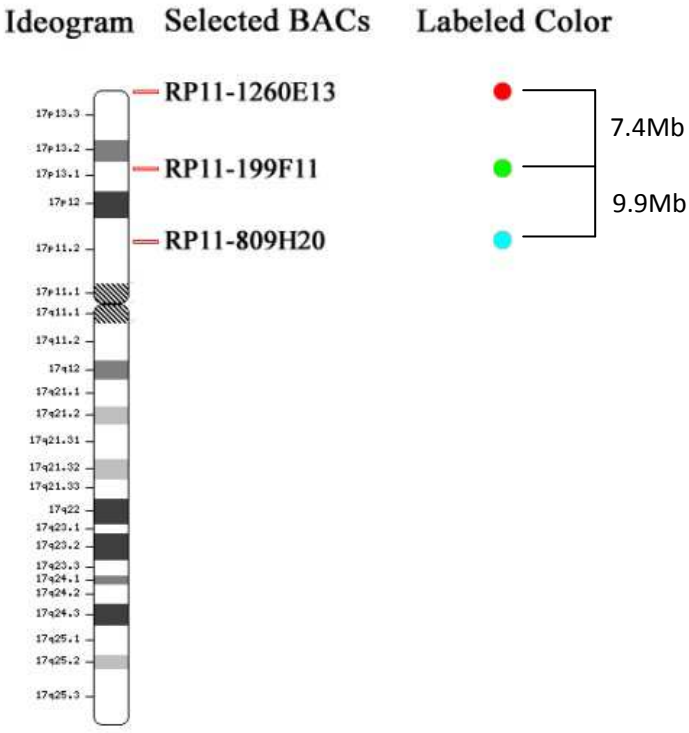


Figure 8. Three BAC clones located on chromosome 17p used in 3-color FISH analysis.

100ng of BAC DNA was labeled with Spectrum Orange-dUTP (for RP11-1260E13; red), Spectrum Green-dUTP (for RP11-199F11; green) and DEAC-dUTP (RP11-809H20; blue).

3.5 Array CGH analysis

3.5.1 CGH arrays

1-Mb resolution whole-genome arrays were provided by the Sanger Institute, UK (http://www.sanger.ac.uk/research/projects/molecularcytogenetics/). Array comparative genomic hybridization (Array-CGH) was performed as described (Fiegler et al 2003). Briefly, 3040 BAC/PAC clones covered each chromosome except the short arms of the acrocentric chromosome at an approximate spacing of 1 Mb were selected from libraries held at the Wellcome Trust Sanger Institute. *Drosophila* BAC clones were obtained from the RPCI-98 library (http://www.chori.org/bacpac/) and used as nonspecific hybridization controls. The clone details can be obtained from the Ensembl human genomic data (http://www.ensembl.org/Homo_sapiens/Cytoview). DNA isolated from BAC/PAC clones was amplified using DOP-PCR and spotted in duplicate onto 3-D link activated slides (Motorola, Schaumburg, IL, USA) using a MicroGrid II arrayer (BioRobotics, Boston, MA, USA) (Hurst et al 2004).

3.5.2 Genomic DNA isolation, labelling and hybridization to arrays

Genomic DNA was extracted from the esophageal squamous cell carcinoma cell line SLMT-1 by using a standard proteinase K digestion followed by phenol/chloroform extraction (Proctor et al 1991). Array CGH was performed as described previously (Fiegler et al 2003). In short, tested SLMT-1 cells and referenced normal esophageal tissue cells genomic DNAs (0.45–1.2µg) were labelled with Cy3-dCTP or Cy5-dCTP using random-primer labelling (BioPrime DNA Labelling Kit, Invitrogen Ltd, Paisley, UK). Micro-spin G50 columns (Amersham Biosciences UK Ltd, Chalfont St Giles, UK) were used to remove unincorporated nucleotides. Labelled DNAs were combined, mixed with 135µg of human CotI DNA (Invitrogen Ltd), precipitated using ethanol (EtOH) and resuspended in hybridization buffer (50% formamide, 10% dextran sulphate, 0.1% Tween 20, 2×SSC, 10 mM Tris-HCl pH7.4). After adding 600µg yeast tRNA (Invitrogen Ltd), the samples were denatured at 72°C for 10 min and incubated at 37°C for 60 min. The well was formed by applying a rubber cement ring around the array (size 2 cm ×3 cm). Then 140µl of hybridization buffer containing denatured herring sperm DNA (880µg) (Sigma) and CotI DNA (135µg) was added to prehybridize the slides. The slides were then incubated at 37°C for 60 min in a humid chamber. Then

most of the pre-hybridization solution was removed, followed by addition of prehybridised genomic DNA. The slide was placed in a slide mailer containing Whatmann 3MM paper saturated with 2x SSC and 20% formamide, sealed with parafilm and incubated on a rocking platform (5 r.p.m.) at 37°C for 48 hrs. Finally, slides were washed in PBS/0.1% Tween 20 for 10 min at room temperature, in 50% formamide/2×SSC for 30 min at 42°C, and in PBS/0/1% Tween 20 for 10 min at room temperature, and then dried by centrifugation at 150g for 5 min.

3.5.3 Image acquisition and data analysis

All previously treated slides were scanned under an Axon 4000B scanner (Axon Instruments, Burlingame, CA, USA). UCSF SPOT software was used to define spots and calculate background subtracted fluorescence intensities of the images (Jain et al 2002). A custom-designed Excel spreadsheet was established with the previously calculated fluorescence intensities. By using the spreadsheet, (1) fluorescence intensity values below the *Drosophila* control spots were excluded, (2) differences in overall signal intensity between Cy3 and Cy5 channels were adjusted by normalizing all signal intensities to a 1 : 1 ratio on autosomal chromosome clones, (3) mean log2 (test/reference) ratios for duplicate spots (discarding data with >20%

difference) were calculated and (4) graphical outputs in the form of individual chromosome plots of mean log2 ratio against distance along the chromosome (Mb) were produced. Ensembl database (release 46) was used for mapping distances in the spreadsheet. Gains or losses of chromosome copy numbers were defined when the normalized mean log2 ratio was above or below ±0.2 respectively. If the region's normalized mean log2 ratio was above 1.2, it was classified as an amplificatory region.

3.6. Gene expression study

3.6.1. Chromosomal positions of the candidate genes

Based on the chromosomal position of the HSR as determined by the 3-color FISH and array-CGH analyses, together with the published chromosomal data available at the Ensembl human genomic data release 46 (http://www.ensembl.org), the known genes located within the region of interest are marked in Figure 9. Those genes known to be tumor suppressors were excluded in this expression study. The corresponding gene coding sequences (CDS) are also available from the NCBI (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/) Genbank database with the following identities: *JC-1* (accession no.: NM_003585); *NP3694* (accession no.: NM_001013672); *Fam101B* (accession no.: NM_182705); *Fam57A* (accession no.: NM_024792); *Q9NRE4* (accession no.: AF229804); *RNMTL1* (accession no.: NM_018146); *Q6ZP06* (accession no.: AK130278) and *Timm22* (accession no.: NM_013337). The PCR primers used in the expression analyses were designed by using Primer 3 program (http://frodo.wi.mit.edu/primer3/) and are summarized in Table 3.

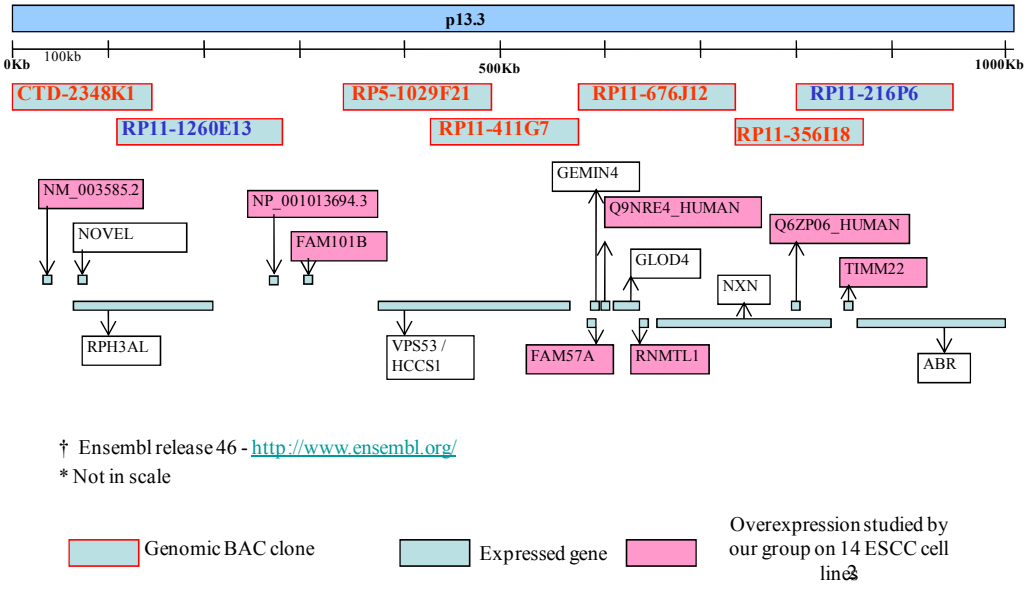


Figure 9. Genes within Chromosome 17p13.3.

A partial genomic map of chromosome 17p13.3 region showing the positions of the genomic BAC clones and the expressed genes. Adopted and modified from the Ensembl human genomic data release 46 (http://www.ensembl.org).

Table 3: Primers used for candidate and house-keeping genes ß-Actin:

ß-Actin-A	5'-GTG GGG CGC CCC AGG CAC CA-3'
ß-Actin-S	5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'
NM3585-b	5'- TGT GTG ACG AGG ACA AAT TC -3'
NM3585-d	5'- GGC CTC AGG TAT GTT TTC AC -3'
NP3694-a	5'- AAC GGA AGG TGA AGA AGA AA -3'
NP3694-c	5'- AGG TTC TCT ATT TCG GCA AA -3'
Fam101B-a	5’-CTGAGCCTACAGGATGTGC-3’
Fam101B-c	5’-GCATTCATCAGAGAGGTCGT-3’
Fam57A-a	5’-CACCGACTGCGTGATGAT-3’
Fam57A-d	5’-CACAGCAGACAGAACCAGTAG-3’
Q9NRE4-a	5'- ACA AAG GAA ACC AAA CCT CA -3'
Q9NRE4-c	5'- TCT TCA TAA CAT TCG CCA AA -3'
RNMTL1-a	5’-GAGAGGTGGTGGAACAGAAG-3’
RNMTL1-c	5’-CACATAGACCCGAGTGTCAG-3’
Q6ZP06-e	5’-TCT GAG TGG TGA AAG GGT CT-3’
Q6ZP06-h	5’-ATA GGG TTT CGC TAT GTT GC-3’
Timm22-a	5’-AGGAGCAGAAGATGATCGAG-3’
Timm22-c	5’-CAGCCACTGATGACACTGTT-3’

3.6.2 RNA extraction

Total RNA was extracted from the cell lines and patients' specimens by using RNeasy Mini Spin Columns Kit (Qiagen). Briefly, the cell lines were harvested as a cell pellet from 75ml culture flasks and 350µl buffer RLT (Qiagen) was added. For the frozen tissue samples and cell pallets, homogenization was performed by vortexing to lyse the cells; then 350µl 70% ethanol was added into the cell lysate with pipetting to further release RNA. The total 700µl mixture including precipitates was then pipetted into an RNeasy Mini spin column (Qiagen) which contains an RNA bounding RNeasy silica membrane. Centrifugation was then performed for the column under 8,000g for 15 seconds. After discarding the flow-through, 700µl RW1 (Qiagen) was added and another 15 seconds of centrifugation at 8,000g was applied. The filtrate together with a collection tube was then discarded. To further purify the RNA extract, salts and contaminants remaining on the membrane were washed off twice by 500µl RPE buffer (Qiagen) using 8,000g centrifugation for 15 seconds and 2 minutes respectively. Finally, 50µl RNase free water (Qiagen) was added into the RNA trapped spin column followed by centrifugation at 8,000g for 1 minute to elute the bound RNA. Then the extracted RNA from samples was stored at -80°C for later expression analysis.

3.6.3 Reverse transcription

The extracted RNAs from the samples were reversely transcribed into cDNA by using GeneAmp® RNA PCR Core Kit (Applied biosystem, CA, USA). Approximately 1.5 μg RNA was used to synthesize cDNA in a 20μl reaction mixture containing 5mM $MgCl_2$, 1mM of each dNTP, 2.5 μM oligo $d(T)_{16}$ primers, 20 units of RNase inhibitor and 50 units of Moloney murine leukaemia virus (MuLV) reverse transcriptase first at 42˚C for 1 hr, then at 95˚C for 10 min.

3.6.4 Multiplex PCR analysis for gene expression

The expression levels of the candidate genes were examined in the ESCC cell lines and ESCC patient cases by using multiplex PCR analysis, and they were compared with those of the immortalized non-tumor esophageal epithelial cells NE3 or the corresponding non-tumor tissues using β-Actin (Tang et al 2007) as the internal control. The primers used are listed in Table 3. The products were then electrophoresed in a 1.5% agarose gel stained with ethidium bromide (EtBr) and visualized under ultraviolet. The intensities of the PCR products were measured by densitometric analysis using the Quantity One program (Bio-Rad). The expression level would be regarded as overexpression if the (target gene/Actin, tumor)/(target gene/Actin, non-tumor) ratio was larger than 1.2; a ratio between 0.8 and

1.2 would be considered as no change of expression level, while a ratio smaller than 0.8 would be considered as underexpression of the target gene (Fatima et al 2006, Tang et al 2007, Zhou et al 2005). The candidate genes and the primers used for the multiplex PCR expression analyses have been previously described in Table 3.

3.6.5 Immunostaining of *Timm22* on cell lines blocks

Immunostaining was also conducted to examine the protein expression level of *Timm22* in the ESCC cell lines. Seven ESCC cell lines (HKESC-3, HKESC-4, KYSE 30, KYSE 150, KYSE 450, KYSE 510 and KYSE 520) and two non-tumor esophageal epithelial cell lines (NE1 and NE3) were tested in this part of experiment. The cell line paraffin blocks were prepared using the cell pellets with approximately $5x10^6$ cells that had been formalin-fixed and wax-embedded. 16-µm sections were prepared from the cell line blocks and were stained by using mouse polyclonal antibody to human *Timm22* (Abcam); the dilution factor was 1:500 and standard procedures were used as previously described (Yuen et al 2007). Photos of immunostaining for each sample were taken under light microscope with the magnification of x400. The intensity of the immunostaining signals was evaluated using the number system as follows: -3 (lightest), -2, -1, 0, +1, +2 and +3 (strongest).

3.7. Transfection and transformation study

3.7.1 Cloning of *Timm22*

The entire coding region of the target gene Timm22 (accession no. NM_013337), which showed the second highest overexpression rate in ESCC cell lines, was amplified from the cDNA of NE1 cells by PCR. The primers were: Timm22-k, 5' TGC TTG GGC AGC GAC TGT CA 3' and Timm22-p, 5' ACT GTC CTC CAG AGA GCA GC 3' (Figure 10). The PCR product (668bp) was purified from 1.5% agarose gel by using QIAEX II gel extraction kit (Qiagen) according to the manual's instruction. The PCR product was then cloned into a pGEM®-T Easy cloning vector (Figure 11, Promega) and subcloned into a pcDNA3.1(-) expression vector (Figure 12, Invitrogen™) by using One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells (Invitrogen™) according to manual's instruction. The sequence and orientation of the insert were confirmed by sequencing. The sequencing primers used were: Timm22-m 5'-CTT GGG CAG CGA CTG TCA TG-3' and Timm22-n 5'-AGT CAC CAA GAT GAC CAC AG-3' (Figure 10). The sequencing service was provided by the Centre for Genomic Sciences, Li Ka Shing Faculty of Medicine, The Hong Kong University.

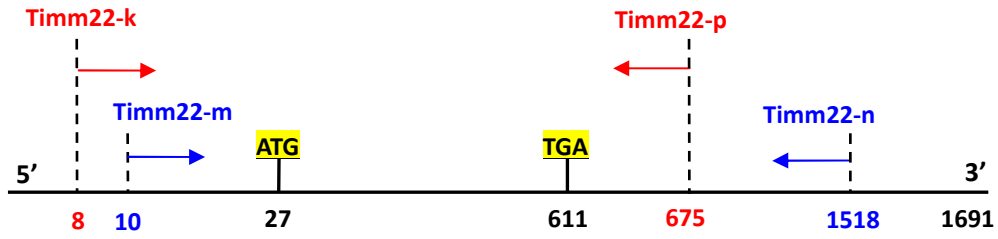


Figure 10. A linear map showing the positions of cloning and sequencing primers used for the *Timm22* study.

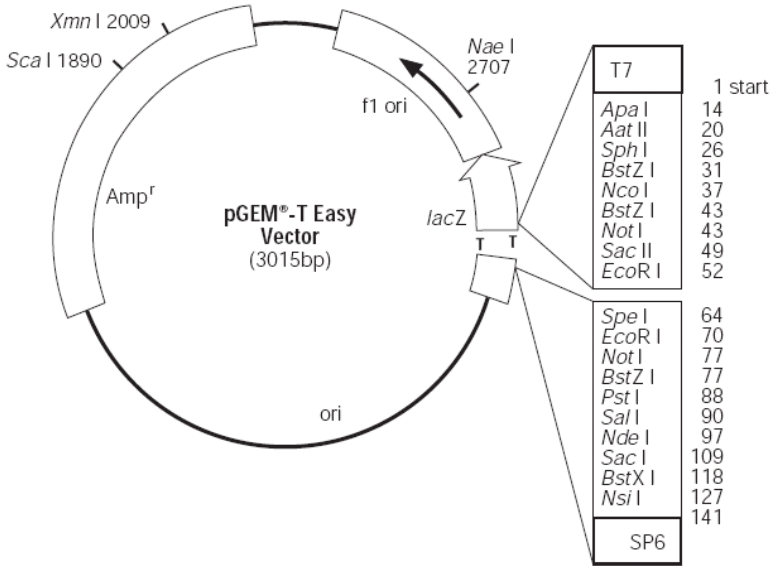


Figure 11. A map of pGEM®-T Vector showing the restriction sites for cloning.
(Adopted from pGEM-T Easy manual, Promega)

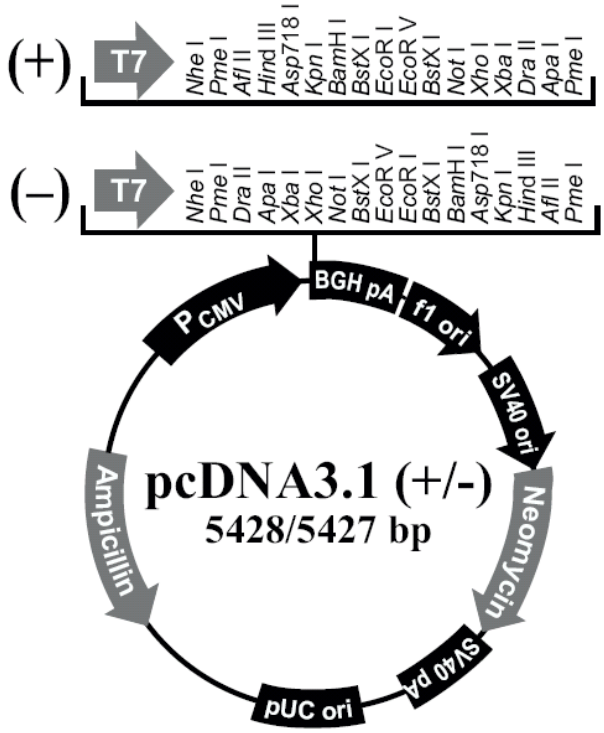


Figure 12. A map of pcDNA3.1(+) and pcDNA3.1(-) vectors showing the restriction sites for cloning.
(Adopted from pcDNA3.1 manual, Invitrogen™)

3.7.2 Transfection with *Timm22* and *JC-1*

The entire coding sequence of *JC-1,* which showed the highest overexpression rate from the ESCC cell lines, was purchased from GenScript (New Jersey, USA) and cloned into a pcDNA3.1(-) vector based on the sequence information from the National Center for Biotechnology Information (accession no. NM_003585). The sequence and orientation of the insert were confirmed by sequencing. The pcDNA3.1(-) vector with *Timm22* or *JC-1* insert was transfected into NIH 3T3 cells (named as 3T3/Timm22 or 3T3/JC-1) by using FuGENE 6 transfection reagent (Roche Applied Science) with 3:1 reagent-to-DNA ratio according to manual's instructions. The mock pcDNA3.1 (-) vector was also transfected into NIH-3T3 cells without any insert and served as negative control (named as 3T3/vec). The H-*ras* V12 gene (Jin et al 2003) cloned into the vector pcDNA3.1(-) was also transfected into NIH-3T3 cells and served as positive control (named as 3T3/*ras*). After 72 hours of transfection, the cells were cultured in the same medium containing G418 (Gibco Corporation) at 100-400μg/ml for selection for 3 months. The expression level of *Timm22* was then confirmed from the transfected cells by RT-PCR analysis as described in the previous section using ß-actin as the control.

3.7.3 Cell proliferation assay

The overexpression of *Timm22* and *JC-1* in 3T3/*Timm22* and 3T3/*JC-1* was confirmed by RT-PCR compared with parental NIH 3T3 and 3T3/vec. After that, the cell growth rates of parental NIH-3T3, 3T3/vec, 3T3/*ras*, 3T3/*Timm22* and 3T3/*JC-1* were compared by MTS assay using CellTiter96® Aqueous One Solution Cell Proliferation assay (Promega) according to the manufacturer's instruction. Briefly, the cells were trypsinized according to a method described in the previous session. The cell number for each kind of cells was then determined by counting using hematocytometer (Boeco, German) under light microscope. Each kind of cells was then separately plated into a 96-well culture plate at a density of 1×10^3 cells per well and maintained in DMEM medium supplemented with 10% FBS at 37^{o}C with 5% CO_2. Viable cells were detected everyday by removing the medium and adding 100µl working MTS solution into each well. The plate was then incubated at 37 ^{o}C for 3 hours. The absorbance of formazan products was then measured at 490nm using a microplate reader (Bio-Rad). Three independent experiments were performed for each kind of cells.

3.7.4 Colony formation assay in soft agar

A 2ml 0.8% agar base layer was prepared in each well of a 6-well plate. The base layer contained cell culture grade water and culture medium in a

1:1 ratio and allowed for setting. After the agar has solidified, parental NIH-3T3, 3T3/vec, 3T3/*ras*, 3T3/*Timm22* and 3T3/*JC-1* cells were harvested from culture flasks separately by trypsinization. Then the top agar layer was prepared by adding 0.8% base agar solution and cell culture media in a 1:1 ratio and containing about 1×10^4 each kind of cell lines respectively. Then the top agar layers containing different cells were placed onto base layer in 6-well plate at 1.5ml per well. After 4 weeks of incubation at 37^{o}C and 5% CO_2 atmosphere, colonies were stained and observed under light microscope. The number of colonies in each well was counted. The experiments were performed in triplicate for each type of cells.

3.7.5 Foci formation assay

1×10^4 of parental NIH-3T3, 3T3/vec, 3T3/*ras*, 3T3/*Timm22* and 3T3/*JC-1* cells were seeded intofive 6-well plates respectively and grown to confluence. The cells were cultured in DMEM medium containing 10% FBS and antibiotics at 37^{o}C and 5% CO_2 atmosphere. The medium was replaced every 2 days. The cells were then stained with 10% methylene blue after washing with 1ml PBS solution. After 14 days, the formation of foci was observed and photographed. Each assay for every cell line was repeated 3 times.

3.7.6 Migration assay

Parental NIH-3T3, 3T3/vec, 3T3/*ras*, 3T3/*Timm22* and 3T3/*JC-1* cells were grown to confluence on 6-well plates at 37 ^{o}C and 5% CO_2 atmosphere cell culture incubator supplied with DMEM medium supplemented with 10% FBS. Then the monolayers of cells were scratch-wounded with an autoclaved 1ml blue plastic pipette tip after a single wash in serum-free medium according to previous methods (Cha et al 1996) and (Hattori et al 2009). Then a cell-free area (wound) approximately 2 mm in width was created. The cells were then allowed to heal the wound in the same medium maintained before in the same condition. Marked areas of wounds were photographed after 12 and 24 hours separately under microscope after washing with PBS.

3.8 Tumorigenicity test in nude mice

Female athymic nude mice were purchased from Laboratory Animal Services Centre, The Chinese University of Hong Kong. All the studies on animals were approved by the Animal Subjects Ethics Subcommittee of the Hong Kong Polytechnic University and The Department of Health of the HKSAR Government. Approximately 1×10^6 cells of each type (Parental NIH-3T3, 3T3/vec, 3T3/*Timm22* or 3T3/*JC-1* cells) were subcutaneously injected into the flank of three female athymic nude mice after selection in G418 for one month. The formations of subcutaneous tumors were observed after 76 days for 3T3/*Timm22* cells or 79 days for 3T3/*JC-1* cells. The tumors were dissected and fixed in 10% formalin for histopathological examination. Total RNAs of the parental NIH-3T3, 3T3/vec, 3T3/*Timm22* and 3T3/*JC-1* cells were extracted before injection and the cDNAs were prepared as previously described. The *Timm22* and *JC-1* expression levels of the samples were examined as described previously using β-actin as the control.

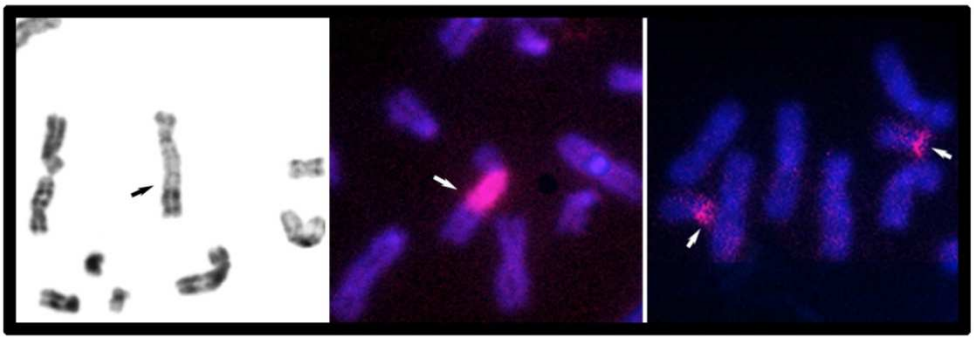
Chapter 4
Results

4.1 Molecular characterization of the homogeneously staining region (HSR) in SLMT-1

4.1.1 G-banding and FISH analyses

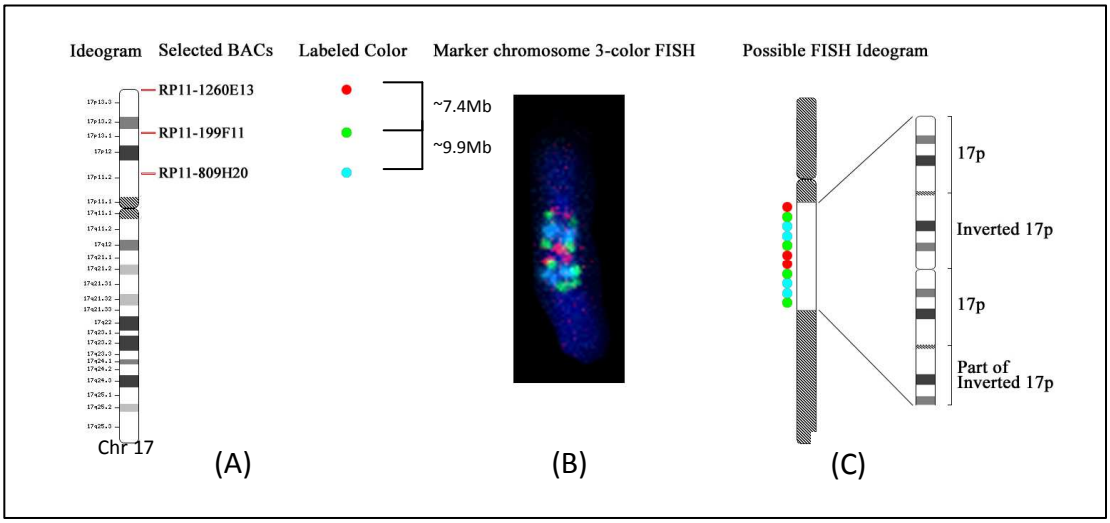
G-banding analysis for ESCC cell line SLMT-1 identified a HSR as a marker chromosome (Figure 13A). After microdissection on the HSR was done, *in vitro* DNA amplification was observed. FISH analysis on SLMT-1 using the chromosome-microdissected DNA probe confirmed the original position of the HSR probe (Figure 13B). To identify the original chromosomal position of the HSR sequence in normal cells, FISH analysis was performed on normal metaphase chromosomes isolated from a normal healthy individual's lymphocytes using the same chromosome-microdissected DNA probe. The result indicated that the chromosomal origin of the HSR was chromosome 17p (Figure 13C). To further locate the chromosomal position of the HSR, "three-color" FISH analysis was further performed on HSR in SLMT-1 using the 17p BAC clones (RP11-1260E13, RP11-199F11 and RP11-809H20; Figure 14A) which covered

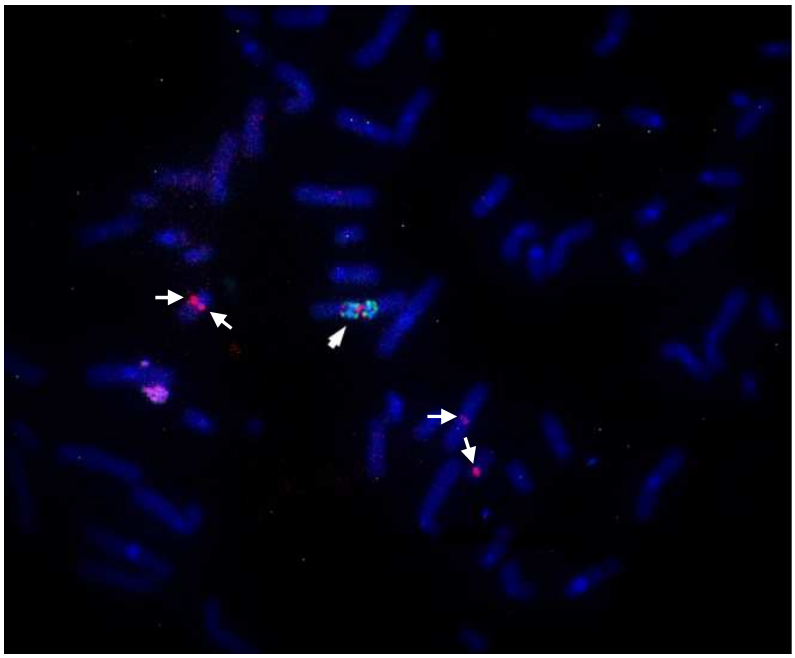
the region of interest according to the result of the previous FISH analyses. The three 17p BAC clones, RP11-1260E13, RP11-199F11 and RP11-809H20, were labeled with Spectrum Orange-dUTP, Spectrum Green-dUTP and DEAC-dUTP (blue) respectively. The signals of the 3-color FISH indicated the pattern of amplification of the 17p region, in which duplication and inversion of the chromosomal segments were involved (Figure 14B and Figure 14C). The ESCC cell line SLMT-1 has been reported (Tang et al 2001b) and confirmed in the present study to be close to a tetraploid karyotype. The overall chromosomal amplification of the 17p BAC clone sequences throughout all the SLMT-1 chromosomes was also evaluated with the overall signals of the 3-color FISH from all the SLMT-1 chromosomes. Figure 14D indicates that there were a total of 7 signal dots of FISH signals which belonged to the RP11-1260E13 BAC clone sequence, but only 4 signal dots were detected for RP11-199F11 and RP11-809H20 BAC clone sequences.



(A) (B) (C)

Figure 13. G-banding and FISH analyses for the homogeneously staining region (HSR) in the ESCC cell line SLMT-1.
(A) G-banding analysis on SLMT-1 showing the HSR (arrow) in a marker chromosome; (B) FISH analysis of the SLMT-1 cells using the chromosome-microdissected probe isolated from the marker chromosome confirming the HSR (arrow); (C) FISH analysis of normal metaphase chromosomes isolated from a normal healthy individual's lymphocytes using the chromosome-microdissected probe showing the chromosomal origin at 17p.





(D)

Figure 14. FISH analysis on the 17p HSR in SLMT-1.
(A) An ideogram of chromosome 17 showing the relative positions of the BAC clones used for the FISH analysis; (B) 3-color FISH signals detected from the marker chromosome with HSR; (C) FISH ideogram showing the arrangement of the BAC clone signals located within the HSR; (D) overall FISH signals as detected from the other chromosomes of SLMT-1.

4.1.2 Array-CGH analysis

Figure 15 shows the DNA copy numbers gain and loss in the 17p region of SLMT-1 as detected by array-CGH analysis. The positive numbers on the Y-axis denoted the signals of overrepresentation of DNA copy of SLMT-1 and the negative numbers denoted the signals of underrepresentation compared with the normal control (male DNA). Figure 15 shows that the most 5' terminal clone of chromosome 17p (RP11-216P6; please refer to Figure 9 for its genomic position) included in this array-CGH analysis showed a minor DNA amplification.

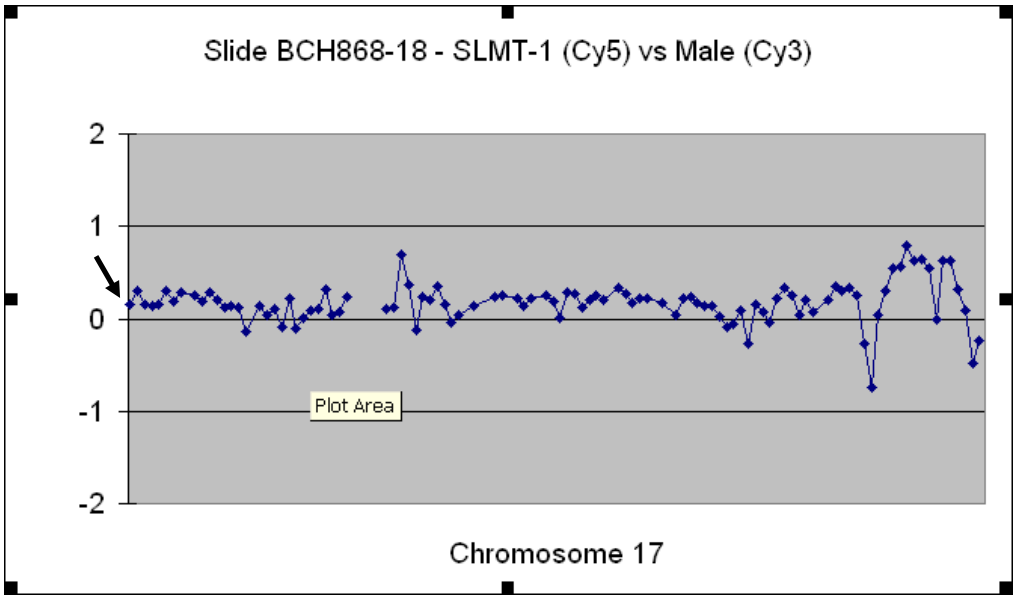


Figure 15. Array-CGH analysis of SLMT-1.
Each plot in the graph indicates one BAC clone sequence starting from the 5' side of the chromosome 17p. The most 5' BAC clone is RP11-216P6 (arrowed) and its genomic position is shown in Figure 9.

4.2 Gene expression study

A total of 14 ESCC cell lines were examined in this study. Four of them are of Chinese origin (HKESC-1, HKESC-2, HKESC-3 and HKESC-4) and the other ten are of Japanese origin (KYSE 30, 70, 140, 150, 180, 270, 410, 450, 510 and 520). The RNA expression levels of the eight non-tumor suppressor genes (Figure 9) were examined by multiplex PCR analysis on the 14 ESCC cell lines using ß-actin expression as the internal control. The percentages of overexpression of all the studied genes are summarized in Table 4. Among the 8 genes examined, *JC-1* showed the highest percentage of overexpression in 11 out of 14 (78.6%) ESCC cell lines compared with NE3. The other 21.4% (3/14) of cell lines showed no changes in the *JC-1* expression level (Figure 16). *Timm22* showed the second highest percentage of overexpression (10 out of 14; 71.4%) among the tested ESCC cell lines. The other 28.6% (4/14) of cell lines showed no changes in the *Timm22* expression level (Figure 17). None of the cell lines showed any *RNMTL1* overexpression and only one showed overexpression with *Fam57A*.

Table 4: Summary of results for multiplex-PCR overexpression study in ESCC cell lines.

Cell lines	**17p13.3 genes involved in present study**							
	JC-1	*NP3694*	*Fam101B*	*Fam57A*	*Q9NRE4*	*RNMTL1*	*Q6ZP06*	*Timm22*
HKESC-1	**OE**	NC	UR	NC	**OE**	NC	NC	**OE**
HKESC-2	**OE**	**OE**	NC	UR	NC	NC	**OE**	**OE**
HKESC-3	NC	NC	NC	UR	ND	ND	**OE**	**OE**
HKESC-4	**OE**	**OE**	UR	NC	NC	NC	NC	NC
KYSE30	NC	NC	UR	UR	NC	NC	UR	**OE**
KYSE70	**OE**	NC	**OE**	**OE**	**OE**	NC	**OE**	**OE**
KYSE140	**OE**	**OE**	**OE**	NC	**OE**	NC	NC	**OE**
KYSE150	NC	**OE**	NC	UR	**OE**	NC	UR	NC
KYSE180	**OE**	**OE**	**OE**	NC	NC	NC	**OE**	NC
KYSE270	**OE**	NC	**OE**	NC	NC	NC	NC	**OE**
KYSE410	**OE**	**OE**	**OE**	UR	NC	UR	UR	**OE**
KYSE450	**OE**	**OE**	NC	NC	**OE**	ND	**OE**	**OE**
KYSE510	**OE**	**OE**	NC	UR	NC	NC	UR	**OE**
KYSE520	**OE**	**OE**	UR	UR	UR	NC	UR	NC
OVERALL (%)	11/14 (78.6%)	9/14 (64.3%)	5/14 (35.7%)	1/14 (7.1%)	5/13 (38.5%)	0/12 (0%)	6/14 (42.9%)	10/14 (71.4%)

Notes: OE: overexpression; NC: no change; UR: underexpression; ND: not done due to poor RNA quality.

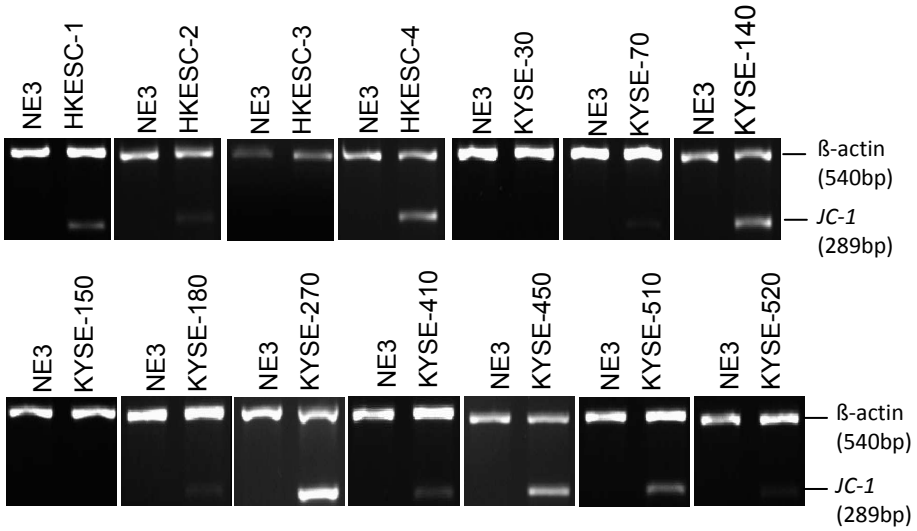


Figure 16. Multiplex RT-PCR analysis of *JC-1* expression level in 14 ESCC cell lines compared with NE3.
Eleven cell lines showed overexpression of *JC-1*: HKESC-1, HKESC-2, HKESC-4, KYSE70, 140, 180, 270, 410, 450, 510 and 520. All the other cell lines showed no overexpression compared with NE3.

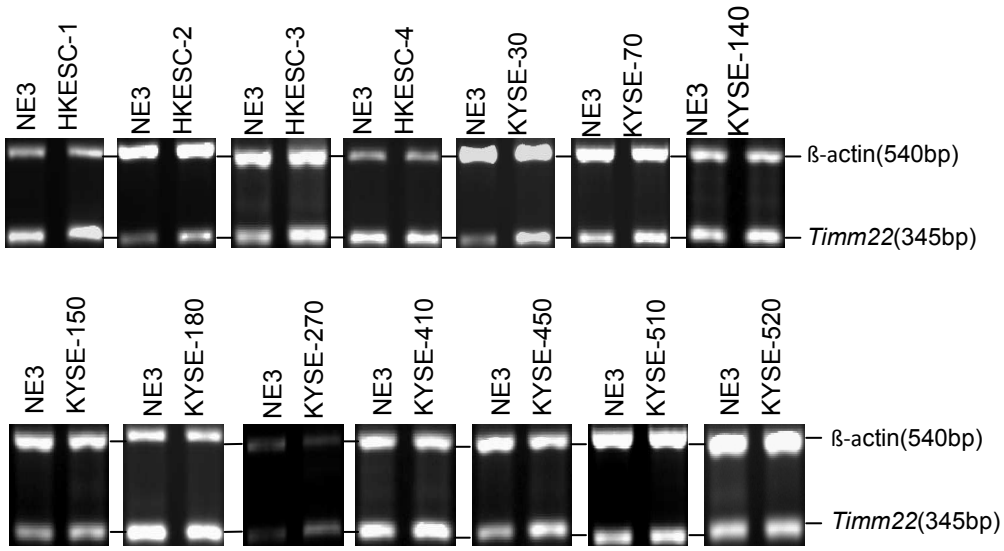


Figure 17. Multiplex RT-PCR analysis of *Timm22* expression level in 14 ESCC cell lines compared with NE3.

Ten cell lines showed overexpression of *Timm22*: HKESC-1, HKESC-2, HKESC-3, KYSE30, 70, 140, 270, 410, 450 and 510. All the other cell lines showed no overexpression compared with NE3.

4.3 Molecular characterization of *Timm22* and *JC-1* in human ESCC

4.3.1 *Timm22* and *JC-1* overexpression in ESCC patient samples

Twenty-nine paired patient tissue specimens (tumor and non-tumor) were examined for *Timm22* overexpression using the RT-PCR approach as previously described and the clinicopathological features are listed in Table 5. There was 1/29 case in stage I (3.4%), 11/29 cases in stage II (37.9%), 14/29 in stage III (48.3%) and 3/29 in stage IV (10.3%). According to the degree of differentiation, 10/29 were well differentiated (34.5%), 13/29 were moderately differentiated (44.8%) and 6/29 were poorly differentiated (20.7%). Among these patient cases, 55.2% (16/29) of the specimens showed at least 1.2-fold *Timm22* overexpression and only 6.9% (2/29) of them showed underexpression, while the other 37.9% (11/29) showed no change in the *Timm22* expression level compared with the corresponding non-tumor samples (Figure 18). According to the clinicopathological features of the cases and the *Timm22* overexpression profiles, it was observed that overexpression of *Timm22* was found more commonly in patients with stage II (8/16; 50%), stage III (6/16; 37.5%) tumors and with moderately differentiation (9/16; 56.3%).

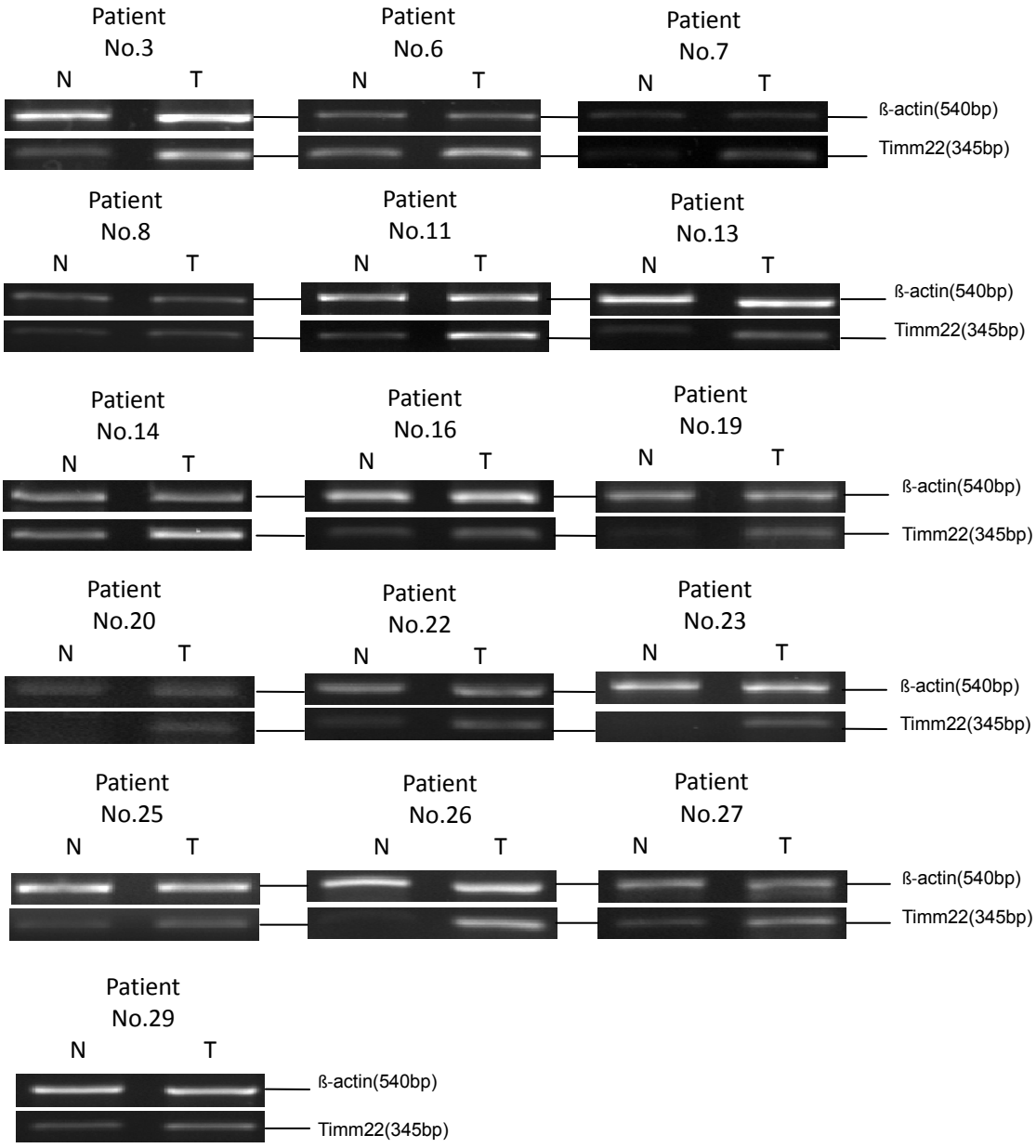


Figure 18. Results of overexpression of the multiplex RT-PCR analysis for *Timm22* expression in ESCC specimens.

N: non-tumor esophagal epithelium cells; T: ESCC tissue. The patient no. could be referred in Table 5. ß-actin was an internal control.

Table 5. Summary of *Timm22* expression studies and the pathological data of ESCC patients.

ESCC Patient no.	*Timm22* expression status	Age/Sex	TNM Stage	Pathologic Staging	Differentiation
1	NC	70/M	T3N0M1b	IVb	Poor / Undifferentiated
2	NC	70/M	T3N0M0	IIa	Well
3	**OE**	60/M	T3N1M0	III	Poor / Undifferentiated
4	UR	62/M	T4N0M0	III	Well
5	UR	74/M	T1N0M0	I	Moderate
6	**OE**	63/M	T3N0M0	IIa	Moderate
7	**OE**	48/M	T3N0M0	IIa	Moderate
8	**OE**	62/M	T4N1M0	III	Moderate
9	NC	67/M	T4N0M0	III	Poor / Undifferentiated
10	NC	62/M	T4N1M0	III	Moderate
11	**OE**	47/M	T3N1M0	III	Moderate
12	NC	55/M	T3N1M0	III	Well
13	**OE**	73/F	T3N0M0	IIa	Well
14	**OE**	70/M	T3N1M0	III	Poor / Undifferentiated
15	NC	47/M	T3N1M0	III	Moderate
16	**OE**	73/M	T3N1M0	III	Moderate
17	NC	57/F	T4N0M0	III	Well
18	NC	69/M	T3N0M0	IIa	Poor / Undifferentiated
19	**OE**	58/M	T3N0M0	IIa	Moderate
20	**OE**	68/M	T3N0M0	IIa	Moderate
21	NC	49/M	T3N0M0	IIa	Well
22	**OE**	53/F	T3N0M0	IIa	Poor / Undifferentiated
23	**OE**	53/M	T4N1M1a	IVa	Moderate
24	NC	62/M	T4N0M0	III	Well
25	**OE**	52/M	T4N0M0	III	Well
26	**OE**	66/F	T3N0M0	IIa	Well
27	**OE**	72/M	T3N0M0	IIa	Well
28	NC	61/M	T3N1M0	III	Moderate
29	**OE**	74/M	T4N1M1b	IVb	Moderate

Thirty-one ESCC patient tissue specimens with clinicopathological data were examined for *JC-1* overexpression compared with the corresponding non-tumor samples as control. The *JC-1* overexpression is shown in Figure 19. Among the 31 specimens, only one (3.2%) showed *JC-1* overexpression (patient no. 1), which was collected from a 63-year-old male patient at stage IIa and had moderately differentiated ESCC. Another ESCC patient case at stage III, with well differentiated carcinoma showed *JC-1* under-expression (3.2%; patient no. 2) and all the other specimens (29 out of 31; 93.5%) showed no changes in *JC-1* expression level. The clinicopathological information of the ESCC patients for the *JC-1* study is summarized in Table 6. There is no significant correlation found between *JC-1* overexpression and clinicopathological features.

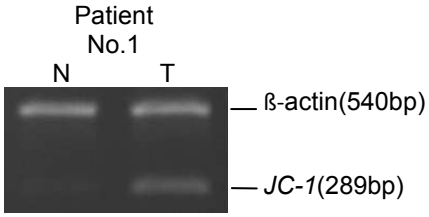


Figure 19. The multiplex RT-PCR analysis for *JC-1* overexpression in ESCC specimens.
ß-actin was used an internal control. N: non-tumor esophagal epithelium cells; T: ESCC tissue. The patient no. is referred in Table 6.

Table 6. Summary of *JC-1* expression study and the pathological data of ESCC patients.

ESCC Patient no.	*JC-1* expression status	Age/Sex	TNM Stage	Pathologic Staging	Differentiation
1	**OE**	63/M	T3N0M0	IIa	Moderate
2	UR	62/M	T4N0M0	III	Well
3	NC	70/M	T3N0M0	Iia	Well
4	NC	70/M	T3N0M1b	Ivb	Poor / Undifferentiated
5	NC	67/M	T4N0M0	III	Poor / Undifferentiated
6	NC	62/M	T4N1M0	III	Moderate
7	NC	74/M	T1N0M0	I	Moderate
8	NC	48/M	T3N0M0	IIa	Moderate
9	NC	62/M	T4N1M0	III	Moderate
10	NC	55/M	T3N1M0	III	Well
11	NC	47/M	T3N1M0	III	Moderate
12	NC	68/M	T3N0M0	IIa	Poor / Undifferentiated
13	NC	68/M	T3N0M0	IIa	Moderate
14	NC	58/M	T3N0M0	IIa	Moderate
15	NC	73/M	T3N1M0	III	Moderate
16	NC	69/M	T3N0M0	IIa	Poor / Undifferentiated
17	NC	57/F	T4N0M0	III	Well
18	NC	74/M	T4N1M0	III	Moderate
19	NC	49/M	T3N0M0	IIa	Well
20	NC	53/F	T3N0M0	IIa	Poor / Undifferentiated
21	NC	61/M	T3N1M0	III	Poor / Undifferentiated
22	NC	53/M	T4N1M1a	Iva	Moderate
23	NC	62/M	T4N0M0	III	Well
24	NC	74/M	T4N1M1b	IVb	Moderate
25	NC	47/M	T3N1M1b	IVb	Moderate
26	NC	72/M	T3N0M0	IIa	Well
27	NC	61/M	T3N1M0	III	Moderate
28	NC	72/M	T4N0M0	III	Well
29	NC	71/F	T3N1M0	III	Poor / Undifferentiated
30	NC	59/M	T3N0M0	IIa	Moderate
31	NC	68/M	T3N0M0	IIa	Moderate

4.3.2 *Timm22* immunostaining

Immunohistochemistry was employed in this study to detect the protein expression level of *Timm22* in NE1, NE3 and the 7 ESCC cell lines (HKESC-3, HKESC-4, KYSE 30, KYSE 150, KYSE 450, KYSE 510 and KYSE 520). As illustrated in Figure 20, *Timm22* protein was detected in both NE1 and NE3 with the intensity score 0. Among the 7 ESCC cell lines, 3 (42.9%; HKESC-3, HKESC-4 and KYSE 30) showed higher signal intensities compared with NE1 and NE3. The other 4 cell lines (57.1%; KYSE150, KYSE450, KYSE510 and KYSE 520) showed lower signal intensities compared with NE1 and NE3.

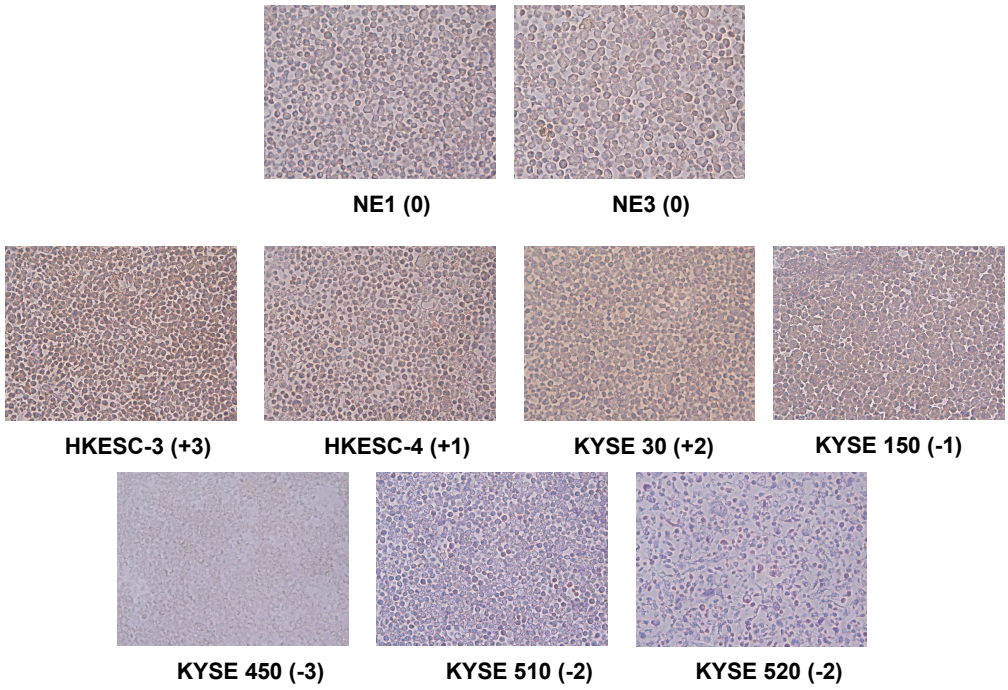


Figure 20. The immunostaining results for NE1, NE3 and the 7 ESCC cell lines using mouse polyclonal antibody to human *Timm22* (Abcam).

Photos were taken under light microscope with magnification x400. The scores of the immunostaining intensities are shown in the brackets next to the names of the cell lines.

4.3.3 Cell proliferation assay

Before the cell proliferation assay was performed, *Timm22* and *JC-1* overexpression levels in parental NIH 3T3, 3T3/vec, and 3T3/*Timm22* or 3T3/*JC-1* cells were examined by RT-PCR analysis to confirm the gene overexpression after the transfection of the vectors pcDNA3.1(-)-*Timm22* or pcDNA3.1(-)-*JC-1*. The results are shown in Figure 21 and Figure 22. The mock vector transfected NIH 3T3 cells were also tested in the growth assay so that the beneficial effect of the vector could be ruled out. The proliferation rate of *Timm22* in 3T3/*ras* is the highest and is comparable to 3T3/*Timm22*, and higher than those of parental NIH 3T3 and 3T3/vec cells (Figure 23). Similar results were shown for *JC-1* overexpressed cells. The proliferation rate in 3T3/ras was the highest, and comparable to 3T3/ *JC-1*, and higher than those of parental NIH 3T3 or 3T3/vec cells (Figure 24).

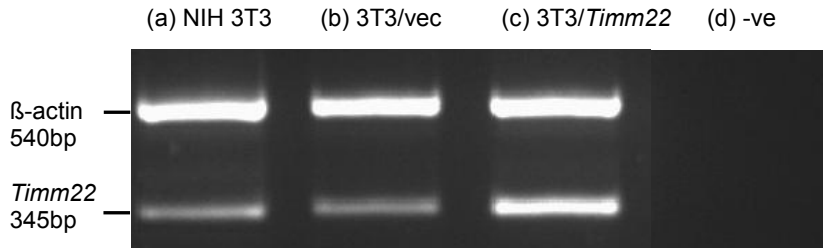


Figure 21. RT-PCR analysis of parental NIH 3T3, 3T3/vec, 3T3/*Timm22* cells and negative control.

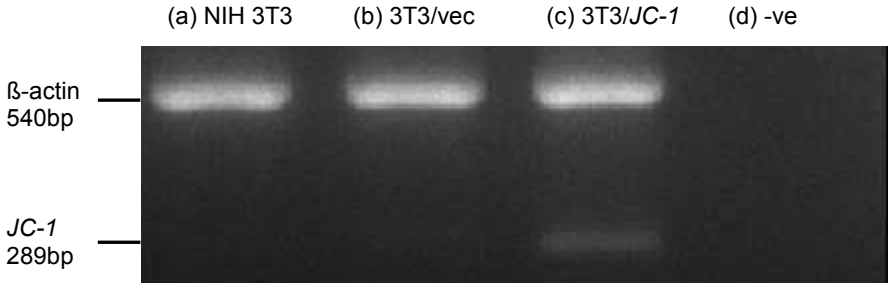


Figure 22. RT-PCR analysis of (a) parental NIH 3T3, (b) 3T3/vec, (c) 3T3/*JC-1* cells and (d) negative control.

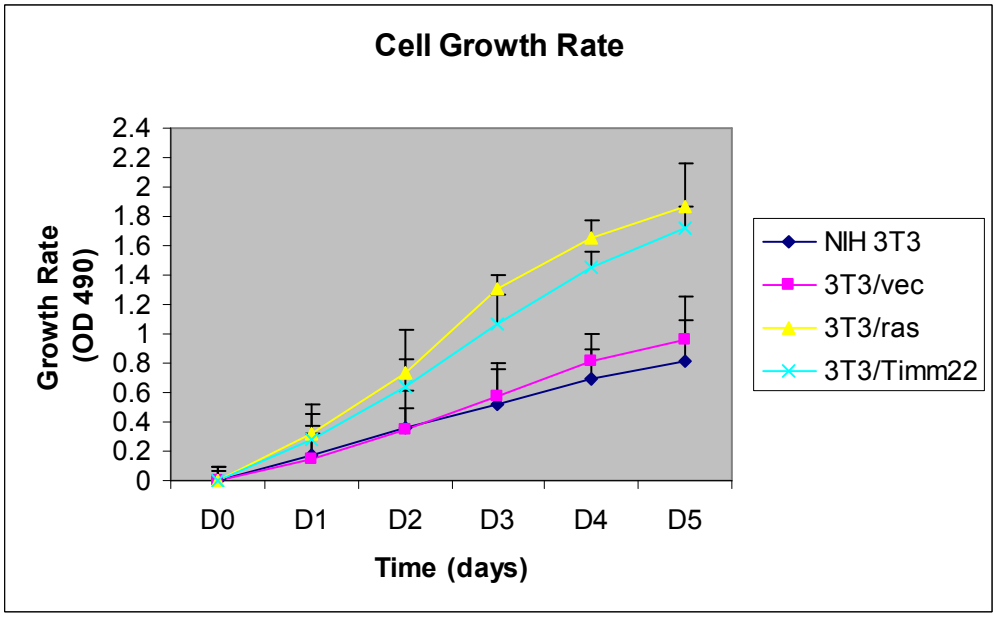


Figure 23. The proliferation properties of parental NIH 3T3, 3T3/vec, 3T3/*Timm22* and 3T3/*ras* cells. The results are presented as mean ± s.d. of three independent experiments.

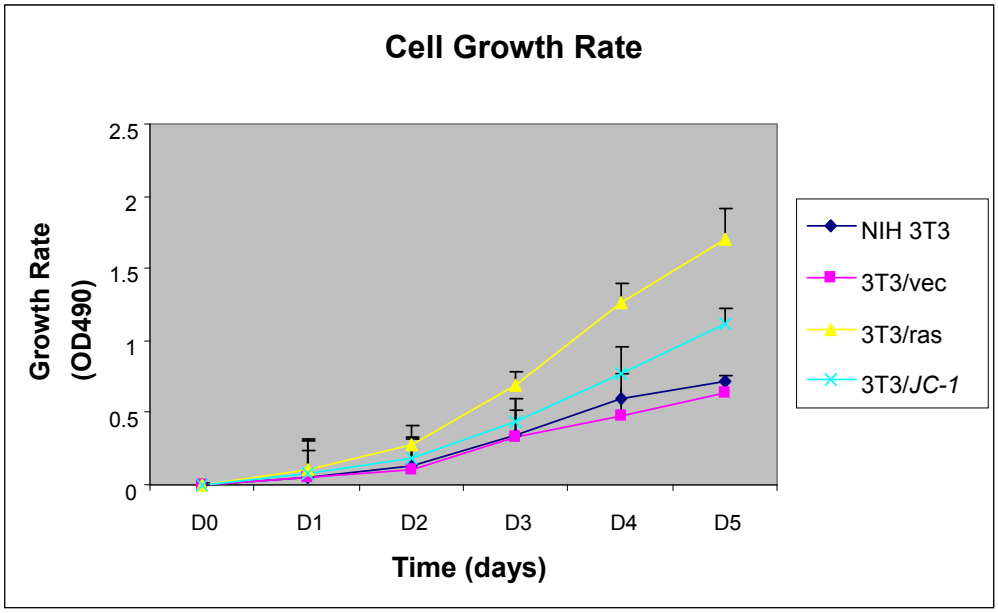
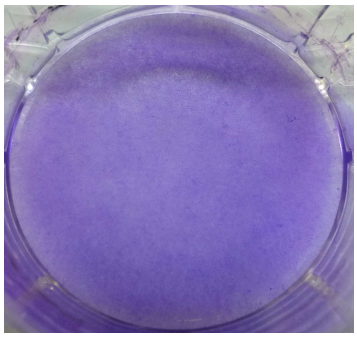


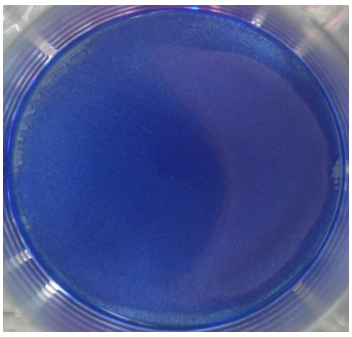
Figure 24. The proliferation properties of parental NIH 3T3, 3T3/vec, 3T3/*JC-1* and 3T3/*ras* cells.
The results are presented as mean ± s.d. of three independent expreiments.

4.3.4 Foci formation assay

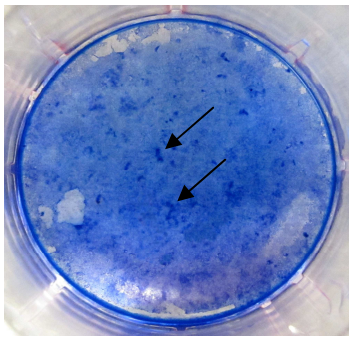
The growth abilities for the target-genes transfected cells were examined by the foci formation assay. The 3T3/*Timm22* and 3T3/*ras* cells showed significant numbers of foci after 2 weeks of incubation with confluent growth. However, the parental NIH 3T3 and 3T3/vec cells did not show any significant foci formation (Figure 25). Similar results were observed in *JC-1* overexpressed NIH 3T3 cells. 3T3/*JC-1* and 3T3/*ras* cells formed significant numbers of foci after 2 weeks of incubation. However, the parental NIH 3T3 and 3T3/vec cells did not show any significant foci formation (Figure 26).



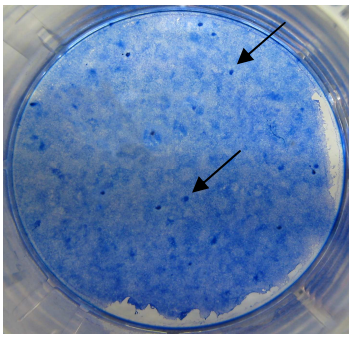
(a) NIH 3T3



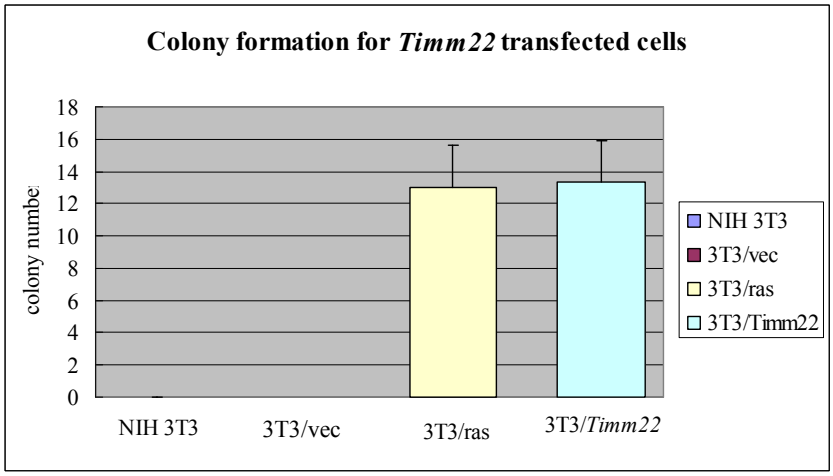
(b) 3T3/vec



(c) 3T3/*ras*



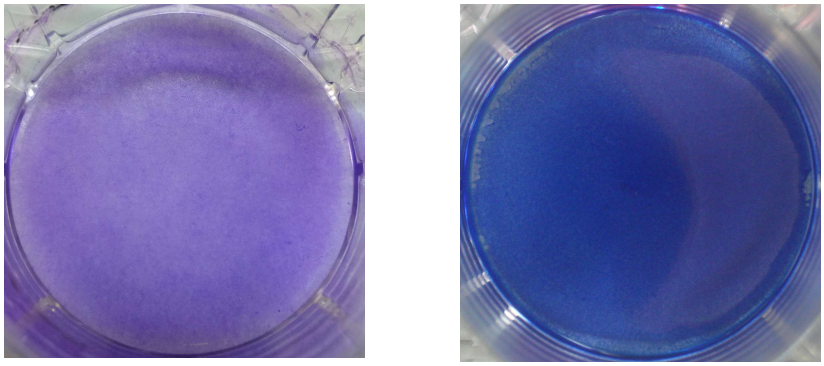
(d) 3T3/*Timm22*



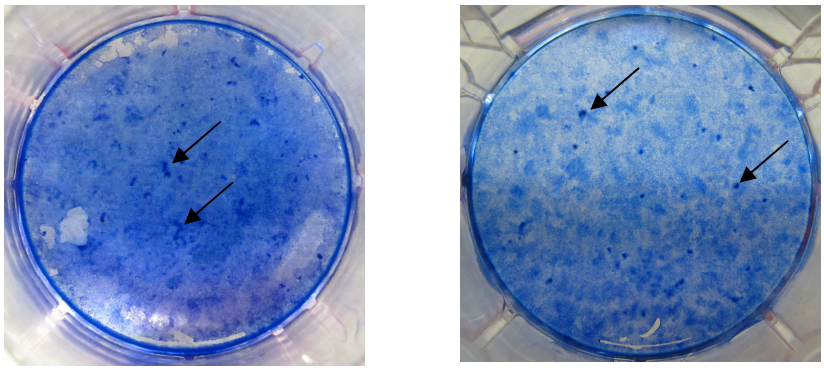
(e)

Figure 25. Foci formation assay for parental (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/*Timm22* cells.

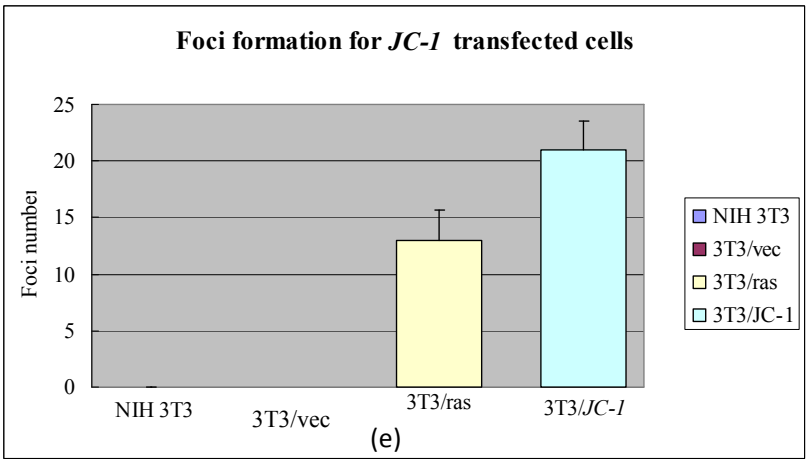
NIH 3T3 cells with *Timm22* overexpression and those transfected with pcDNA3.1-H-*ras* showed the formation of foci (arrows), but not in parental NIH 3T3 cells or mock vector control. Photos were taken after 2 weeks of incubation. (e) 3T3/*Timm22* and 3T3/*ras* cells showed an obvious increase in the quantity of foci.



(a) NIH 3T3 (b) 3T3/vec



(c) 3T3/*ras* (d) 3T3/*JC-1*



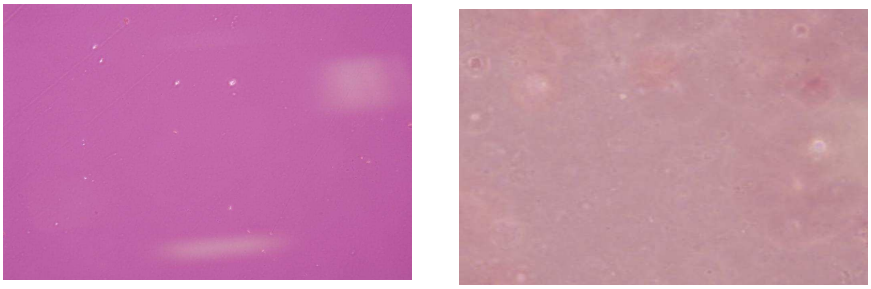
(e)

Figure 26. Foci formation assay for parental (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/ *JC-1* cells.

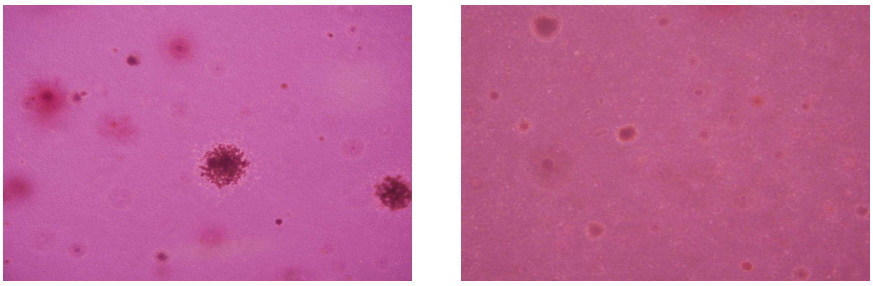
NIH 3T3 cells with *JC-1* overexpression and those transfected with pcDNA3.1-H-*ras* showed formation of foci (arrows), but not the parental NIH 3T3 cells or mock vector control. (e) The foci formation abilities obviously increased in 3T3/*ras* and 3T3/ *JC-1* cells. Photos were taken after incubation for 2 weeks.

4.3.5 Soft agar assay

The anchorage independent growth ability was detected by the soft agar assay. 3T3/*ras* and 3T3/*Timm22* cells showed significant numbers of colonies after 4 weeks of incubation. However, no colony was observed in parental NIH 3T3 or 3T3/vec cells (Figure 27). Similar results were observed for *JC-1* overexpressed cells. Significant numbers of colonies were formed after 4 weeks of incubation for 3T3/*ras* and 3T3/*JC-1* cells while no colony was observed in parental NIH 3T3 or 3T3/vec cells (Figure 28).



(a) NIH 3T3 (b) 3T3/vec



(c) 3T3/*ras* (d) 3T3/*Timm22*

(e)

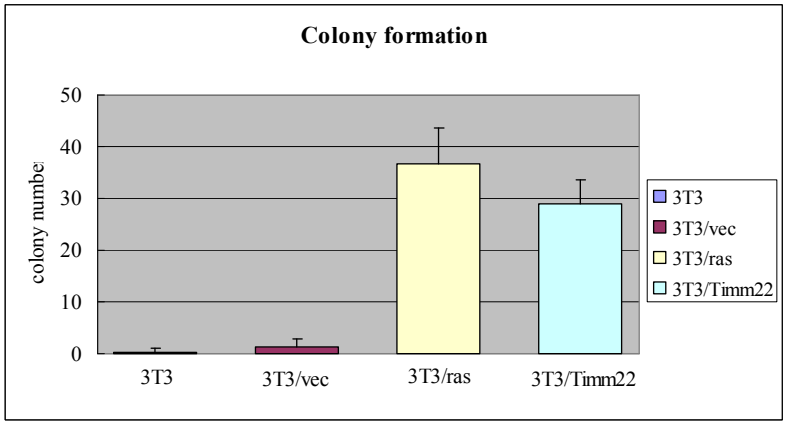
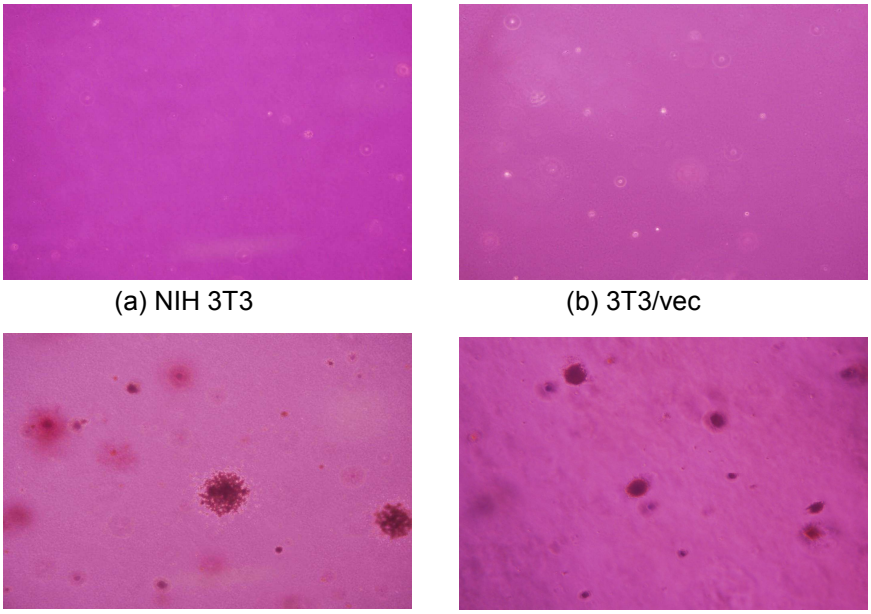
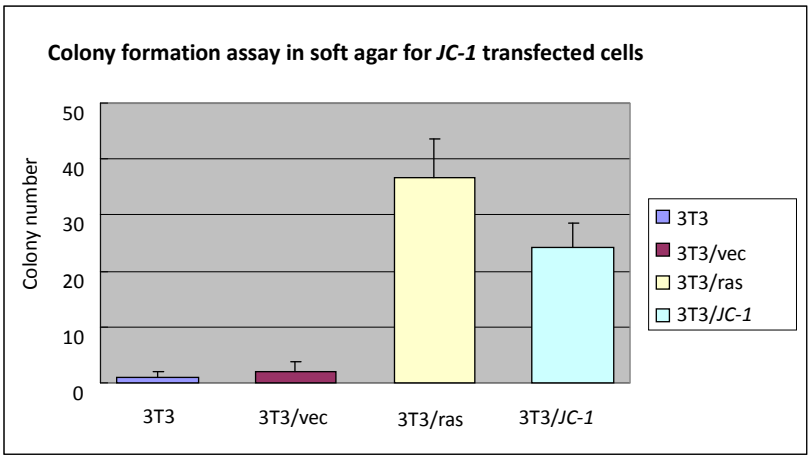


Figure 27. Colony formation as detected by soft agar assay using (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/*Timm22* cells after 4 weeks of incubation.
Original magnification x200. (e) The colony formation abilities are significantly higher in 3T3/*ras* and 3T3/*Timm22* cells.



(a) NIH 3T3 (b) 3T3/vec

(c) 3T3/ras (d) 3T3/*JC-1*



(e)

Figure 28. Colony formation as detected by soft agar assay using (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/ *JC-1* after 4 weeks of incubation. Original magnification: x200. (e) Colony formation abilities are obviously higher in 3T3/*ras* and 3T3/*JC-1* cells.

4.3.6 Cell migration assay

The *in vitro* wound healing assay on NIH 3T3, 3T3/vec, 3T3/*ras* and 3T3/*Timm22* or 3T3/*JC-1* cells was performed on culture plates to detect the effect of *Timm22* and *JC-1* overexpressions on cell migration rates. 3T3/*ras* and 3T3/*Timm22* cells began to migrate over the wound and covered more than half of the wound areas in less than 24 hours. In contrast, the parental NIH 3T3 and 3T3/vec cells acted as negative control and covered less than half of the wound after 24 hours. Thus cell migration was promoted in 3T3/*ras* and 3T3/*Timm22,* but not in NIH 3T3 and 3T3/vec cells (Figure 29). Similar results were shown for *JC-1* overexpressed cells. The cells were covered more than half of the wound areas for 3T3/*ras* and 3T3/*JC-1* cells in less than 24 hours. However, the parental NIH 3T3 and 3T3/vec cells that acted as negative control did not migrate over the wound as fast as 3T3/*ras* and 3T3/*JC-1* cells after 24 hours (Figure 30).

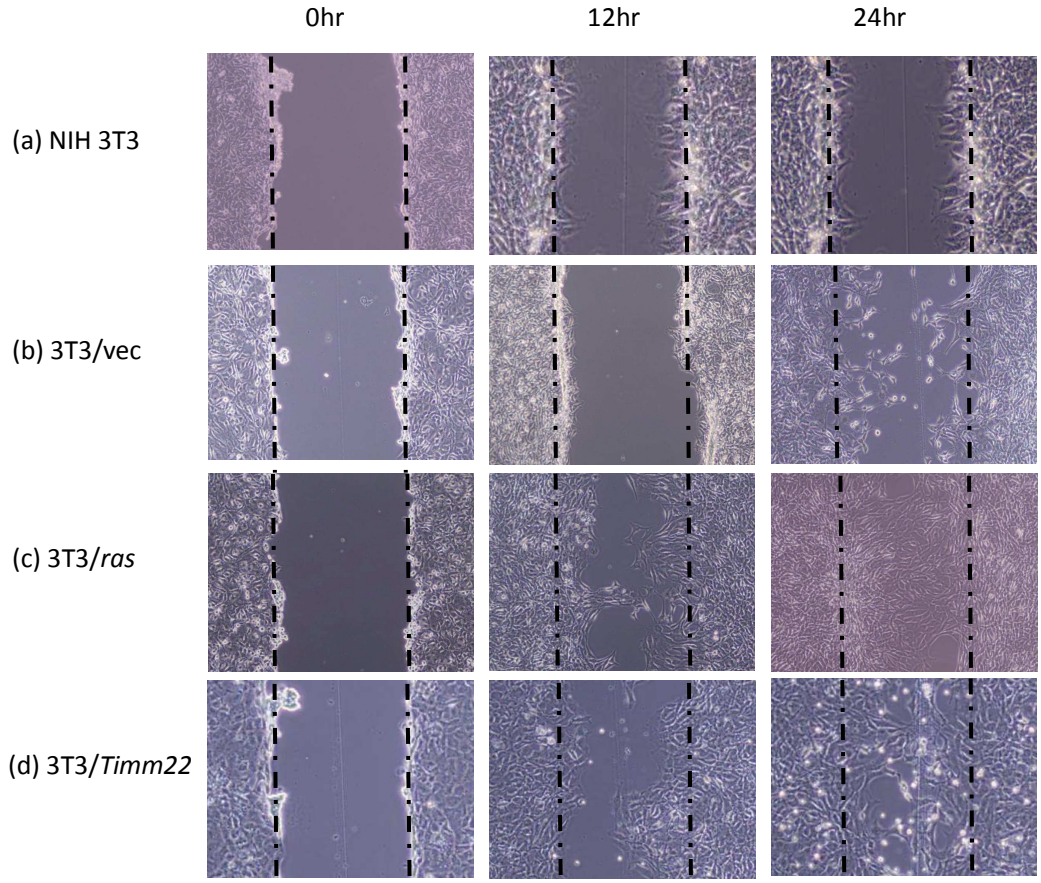


Figure 29. *In vitro* migration assay for (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/*Timm22* cells.
Photos were taken under microscope with original magnification x100.

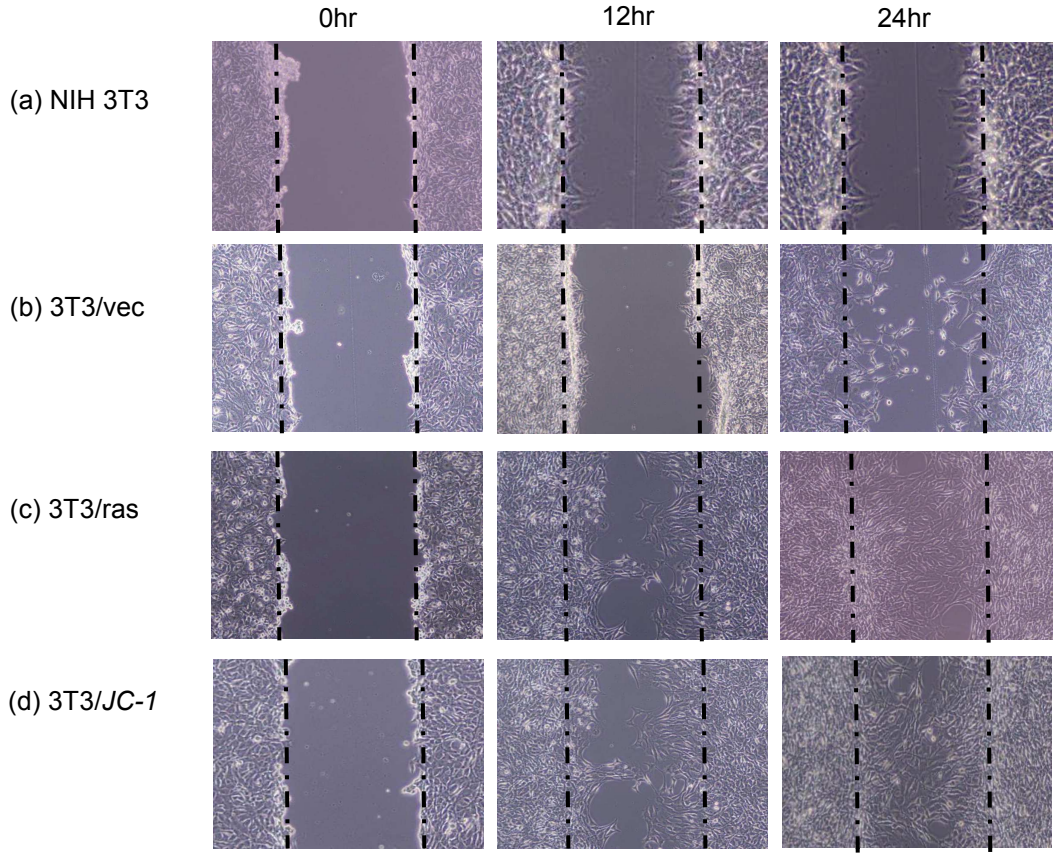
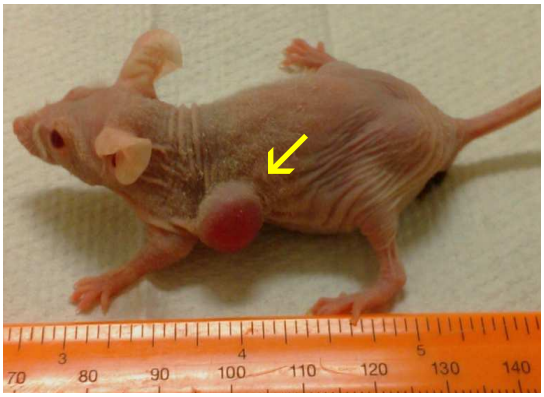


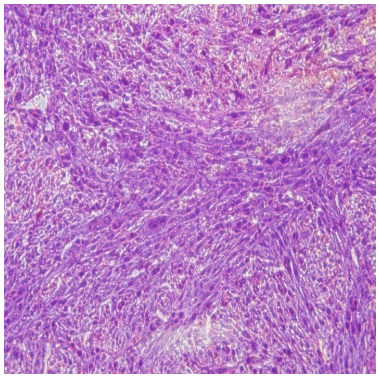
Figure 30. *In vitro* migration assay for (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*ras* and (d) 3T3/*JC-1* cells.
Photos were taken under microscope with original magnification x100.

4.3.7 Tumorigenicity test of *Timm22* in nude mice

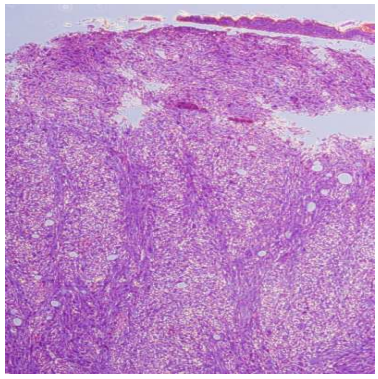
3T3/*ras*, 3T3/*Timm22* or 3T3/*JC-1* cells were subcutaneously injected into the flanks of 9 athymic nude mice, 3 for each cell type to test the *in vivo* tumorigenicity potential of overexpression of *Timm22* and *JC-1*. All the tested mice (3/3) showed subcutaneous tumors at the site of injection with 3T3/*Timm22* cells after 76 days of growth (Figure 31A). Subcutaneous tumors were formed in all the tested mice (3/3) at the site of injection with 3T3/*JC-1* cells after 79 days (Figure 32A). All the mice with injection of 3T3/*ras* cells (3/3) showed the formation of subcutaneous tumor too. However, no tumor was formed in the 3 mice tested with the parental NIH 3T3 or 3T3/vec cells. Histological analysis of the subcutaneous tumors induced by 3T3/*Timm22* showed the features of undifferentiated sarcoma with cellular fascicles of spindle tumor cells with prominent nuclear pleomorphism (Figure 31B). Histological analysis of the subcutaneous tumors induced by 3T3/*JC-1* showed the features of undifferentiated sarcoma with cellular fascicles of spindle tumor cells with prominent nuclear pleomorphism (Figure 32B). RT-PCR analysis demonstrated the overexpression of *Timm22* and *JC-1* in the original 3T3/*Timm22* or 3T3/*JC-1* cells, but not in the parental NIH 3T3 or 3T3/vec cells (Figure 33 and Figure 34).



(A)

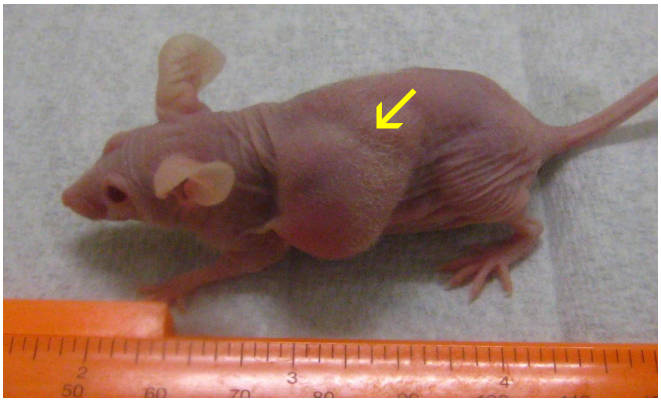


(B)

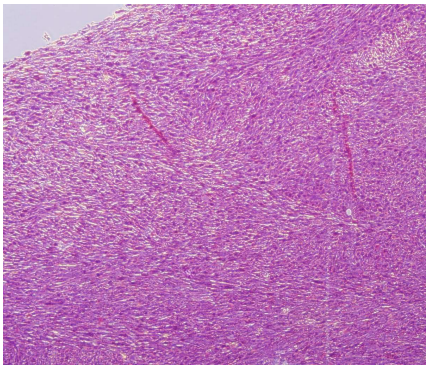


(C)

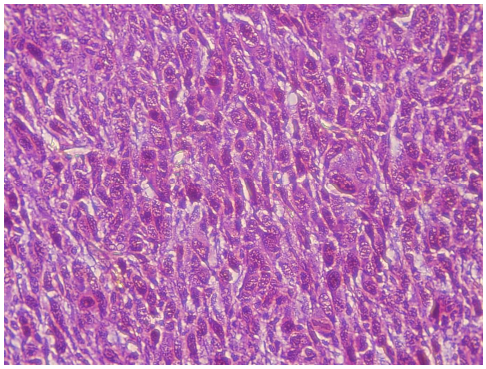
Figure 31. Tumorigenic potential of *Timm22* in nude mice *in vivo*.
(A) Development of a subcutaneous tumor mass (arrow) after the subcutaneous injection of 10^5 *Timm22*-transfected NIH-3T3 cells for 76 days growing. Histological appearance of the subcutaneous tumor mass shows the morphology of undifferentiated sarcoma with cellular fascicles of spindle tumor cells with prominent nuclear pleomorphism, H&E stained. Magnifications: (B) x100; (C) x400.



(A)



(B)



(C)

Figure 32. Tumorigenic potential of *JC-1* in nude mice *in vivo*.
(A) Development of a subcutaneous tumor mass (arrow) after subcutaneous injection of 10^7 *JC-1*-transfected NIH-3T3 cells and 79 days growing. Histological appearance of the subcutaneous tumor mass showing the morphology of undifferentiated sarcoma with cellular fascicles of spindle tumor cells with prominent nuclear pleomorphism, H&E stained. Magnifications: (B) x100; (C) x400.

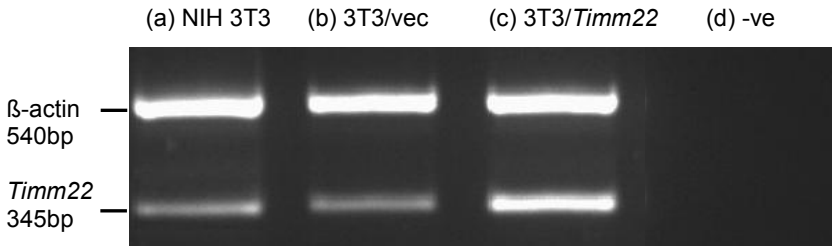


Figure 33. Multiplex RT-PCR analysis for *Timm22* expression in (a) NIH 3T3, (b) 3T3/vec cells, (c) 3T3/*Timm22* cells and (d) negative control.

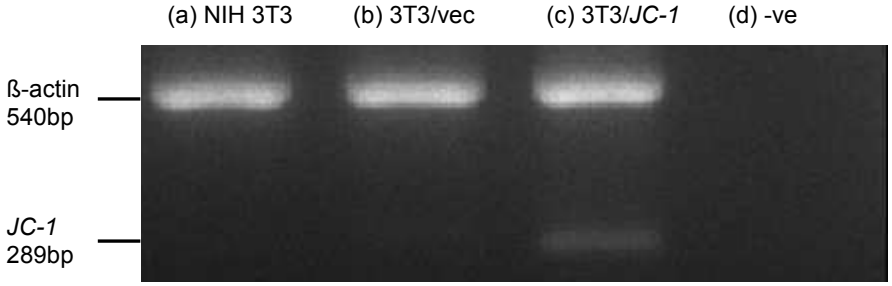


Figure 34. Multiplex RT-PCR analysis for *JC-1* expression in (a) NIH 3T3, (b) 3T3/vec, (c) 3T3/*JC-1* cells and (d) negative control.

Chapter 5
Discussion

5.1 Molecular characterization of the Homogeneously Staining Region (HSR) in human ESCC

It is widely accepted that ESCC is an aggressive malignant cancer with extremely uneven geographical distribution (Jemal et al 2010). The 5-year survival rate of ESCC with multimodality treatment remains at less than 10% (Kwong et al 2004). The survival rate of ESCC in the long run may be improved by understanding more about its molecular pathogenic mechanisms, which hopefully may help identify novel therapeutic targets. Genetic alterations in carcinogenesis of ESCC have been widely studied. However, the detailed molecular pathogenesis of ESCC is still not fully understood. Therefore, the identification of novel genes associated with ESCC can contribute to understanding more about the disease and improve current treatment protocols.

Homogeneously staining regions (HSRs) are chromosomal segments appeared with greatly lengthened and expanded morphologies and are frequently found in the chromosomes of cancer cells (Biedler and

Spengler 1976). A gene or genes that amplified in HSRs are usually included with selective advantages to the progression of the cancer (Bizaril et al 2006). In the present study, a homogeneously staining region (HSR) was first identified in a marker chromosome of unknown origin in SLMT-1 by G-banding analysis. Since there is high incidence of ESCC in Asia that includes Hong Kong (Fatima et al 2006, Tang et al 2007), studies of the chromosomal or molecular features of SLMT-1 of Hong Kong Chinese origin (Tang et al 2001a) may provide new insights about the molecular features of ESCC. FISH analysis was performed on SLMT-1 by using the chromosome-microdissected probe isolated from the marker chromosome to confirm its HSR origin. Following that, another FISH analysis was conducted on the normal metaphase chromosomes isolated from a normal healthy individual's lymphocytes using the same chromosome-microdissected probe to reveal the origin of the HSR, which turned out to belong to chromosome 17p. To further locate the position of the HSR in the chromosome 17p, "three-color" FISH analysis was performed on SLMT-1 using the 17p specific probes, which included RP11-1260E13, RP11-199F11 and RP11-809H20 (Figure 8). The signals of the 3-color FISH indicated the pattern of amplification of the 17p region, in which duplication and inversion of the chromosomal segments were observed (Figure 14B and C). The ESCC cell line SLMT-1 has been reported (Tang et al 2001b) and

confirmed in the present study to be close to a tetraploid karyotype. The overall chromosomal amplification of the 17p BAC clone sequences throughout all the SLMT-1 chromosomes was also evaluated with the overall signals of the 3-color FISH from all the SLMT-1 chromosomes. The results indicated that there were a total of 7 signal dots of FISH signals that belonged to the RP11-1260E13 BAC clone sequence, but only 4 signal dots were detected for RP11-199F11 and RP11-809H20 BAC clone sequences (Figure 14D). However, array-CGH analysis did not show any significant genomic amplification at the RP11-216P6 region. Thus based on the overall results of the "3-color FISH" and array-CGH analyses, the 17p HSR of SLMT-1 showed genomic amplification that started from the most 5' position as denoted by the BAC clone RP11-1260E13 and ended at the BAC clone position RP11-216P6 (Figure 35). Since it has been reported that gene overexpression is usually associated with genomic amplification (Albertson 2006, Gollin 2001), therefore it is very important to further investigate whether the known genes that have been mapped within this 900kb region (RP11-1260E13 to RP11-216P6) showed any significant levels of overexpression in ESCC and how important these genes are with respect to molecular carcinogenesis.

The common feature of 17p amplification in ESCC has also been reported previously. CGH analysis performed on 31 KYSE series (Shinomiya

et al 1999) and 13 TE series (Pimkhaokham et al 2000, Sonoda et al 2004) of ESCC cell lines also indicated the status of chromosomal gain and loss of the 17p region (Figure 36). According to the CGH database (http://www.cghtmd.jp/CGHDatabase/about/introduction_e.htm), most parts of chromosome 17 have chromosomal gains. Other CGH analyses on primary tumors of ESCC in Hong Kong (Kwong et al 2004) and Japan (Shinomiya et al 1999) also showed the chromosomal gains in the 17p region (Figure 37 and Figure 38). Therefore, it is believed that chromosome 17p could be a potential target for harboring oncogene(s), which is the second part of the present study.

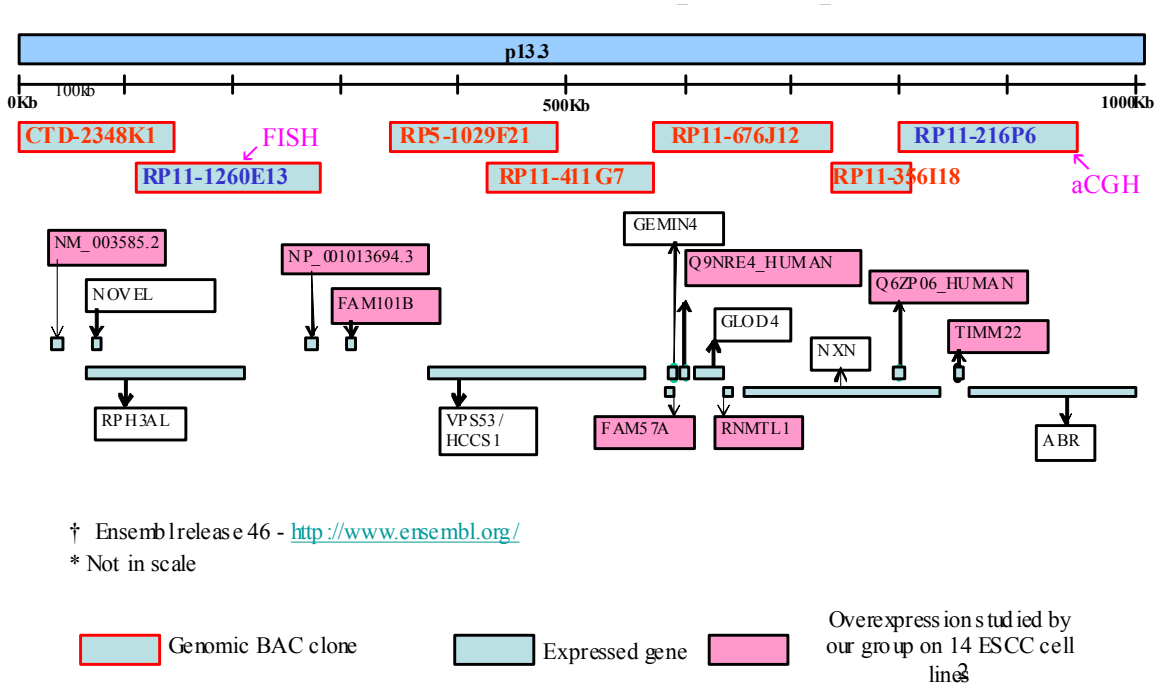


Figure 35. A partial chromosome map of 17p13.3 region showing the positions of the genomic BAC clones and the expressed genes.
Adopted and modified from the Ensembl human genomic data release 46 (http://www.ensembl.org).

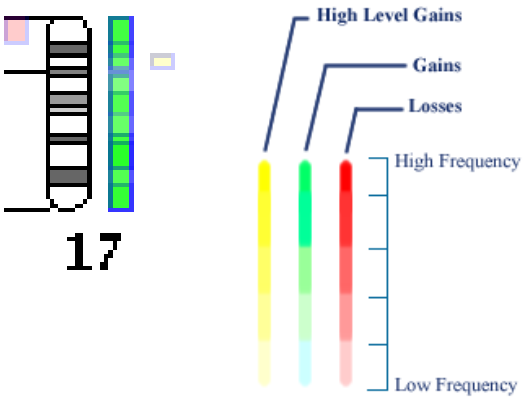


Figure 36. A summary of CGH analyses performed on KYSE (×31) and TE (×12) cell-line series.
Adopted from: CGH Database at http://www.cghtmd.jp/CGHDatabase/about/introduction_e.htm

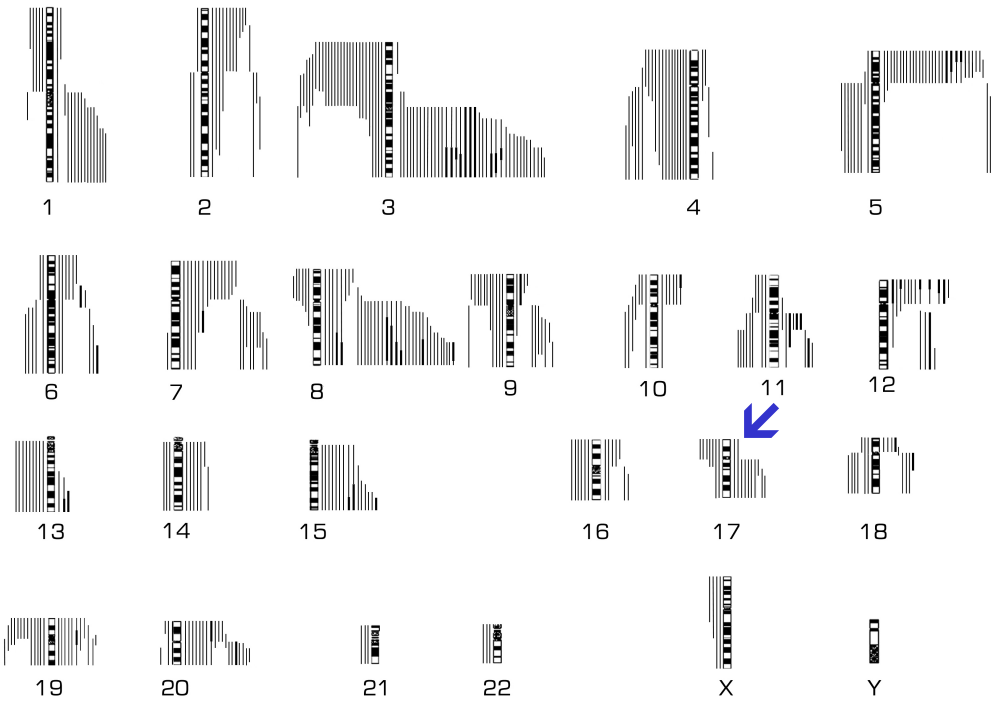


Figure 37. CGH analysis on primary tumors of ESCC in Hong Kong. (Kwong et al 2004)

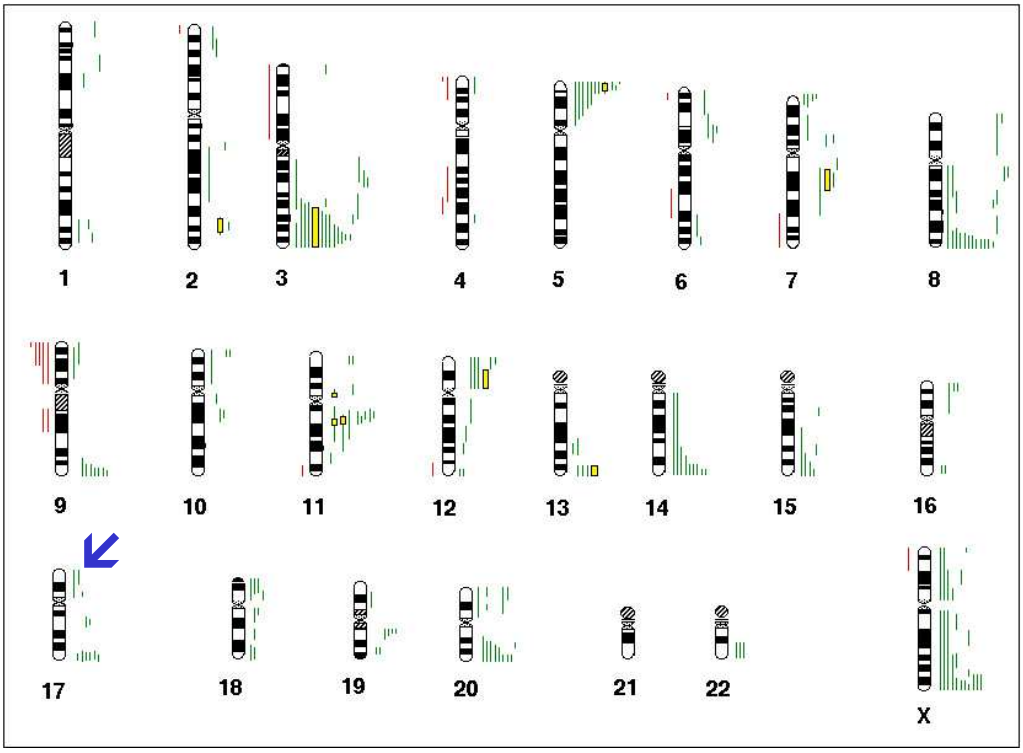


Figure 38. CGH analysis on primary tumors of ESCC in Japan.
(Shinomiya et al 1999).

5.2 Study of RNA expression of the 8 genes in chromosome 17p13.3

Based on the results of FISH and array-CGH analyses, it was believed that the amplicon in HSR was about 900kp and located between the clones RP11-1260E13 and RP11-216P6. According to the genomic data available at Ensembl release 46 (http://www.ensembl.org/), a partial chromosome map of 17p13.3 was constructed and shown in Figure 36. Fifteen protein coding transcripts were mapped within the chromosome region. However, since there are studies indicating that chromosome 17p show high frequency allelic loss in ESCC (Hu et al 2006, Huang et al 2000) and other diseases such as epileptogenesis (Shimojima et al 2010), emphysema (Choi et al 2009) and lung cancer (Konishi et al 1998), therefore those genes that had been reported to have functions of tumor suppressor were not included in the present study of the HSR oncogenes. Among the 15 genes located within the chromosome 17p13.3 region, five of them were proven to be tumor suppressor genes. They are *RPH3AL* (Goi et al 2002, Smith et al 1999), *VPS53* (Smith et al 1999, Xiao et al 2003), *GEMIN4* (Charroux et al 2000, Xiao et al 2003, Zhao et al 2001), *GLOD4* (Wan et al 2004) and *ABR* (Choi et al 2009). *NXN* and *NOVEL* have no available mRNA information. As a result, the 8 non-tumor suppressor genes were investigated in this study for the

discovery of novel oncogenes with higher priority.

The expression level of the 8 genes, *JC-1*, *NP3694, Fam101B, Fam57A, Q9NRE4, RNMTL1, Q6ZP06* and *Timm22*, were examined by semi-quantitative multiplex RT-PCR analysis by comparing with the housekeeping gene *β-actin*. The protocols for the PCR analysis conducted in this study were optimized to ensure the exponential amplification of PCR products and the amount of PCR product was amenable to changes in RNA amount, but not in saturation conditions which reaction substrates turn out to be exhausted and PCR product no longer doubles (Ivinson 1991) and (Harrison and Lidgard 2007). It is well documented that semi-quantitative RT-PCR analysis was well established for studying the expression level of specific genes in ESCC (Fatima et al 2006, Ito et al 2003, Lu et al 2003, Tang et al 2007, Yang et al 2005, Zhou et al 2005). In the present study, the investigations on the expression levels of the eight genes revealed that two of them had over 70% of overexpression in the ESCC cell lines. Among these two genes, *JC-1* showed overexpression in 78.6% (11/14) and *Timm22* showed overexpression in 71.4% (10/14) of the ESCC cell lines and they were further studied for their oncogenic features in later parts of the studies. Although *Timm22* is a known gene with functions previously studied, its roles in the molecular pathogenesis in ESCC, however, was only investigated for the first time in the present study. *JC-1* is a new gene without any known

cellular functions reported and was investigated for the first time.

Overexpression of genes has been widely reported to be relevant in molecular pathogenesis in ESCC. For example, it was found that *cyclin D1*, a nuclear factor, was overexpressed in many ESCC cell lines (Doki et al 1997, Nakagawa et al 1995) and tumor samples from ESCC patients (Chetty and Chetty 1997, Doki et al 1997, Inomata et al 1998, Ishibashi and Lippard 1998, Jiang et al 1992, Jiang et al 1993, Naitoh et al 1995, Roncalli et al 1998, Sheyn et al 1997, Takeuchi et al 1997, Tsuruta et al 1993). Researchers found that overexpression of *cyclin D1* might contribute to the fast growth and enhanced tumorigenesis in ESCC (Baldin et al 1993). Moreover, *ras* gene was found overexpressed in ESCC as a signal transducer (Kim et al 1988). It was suggested that the overexpression of *ras* is related to a higher proliferative status of ESCC cells than their corresponding normal cells (Casson et al 1997). The gene *bcl-2*, which encodes an anti-apoptotic molecule, was also found to be overexpressed in 32-74% of ESCC (Koide et al 1997, Ohbu et al 1995, Ohbu et al 1997, Parenti et al 1997, Puglisi et al 1996, Sarbia et al 1996). Our group also reported two novel genes, *JS-1*(Fatima et al 2006) and *JK-1*(Tang et al 2007), which showed tumorigenic and overexpression properties in ESCC. NIH 3T3 cells with *JS-1* and *JK-1* overexpression were found to have faster growth rates, increased abilities to form colonies in soft agar, increased foci formation in confluence growth and formation of high grade

sarcoma in athymic nude mice after subcutaneous injection of *JS-1* or *JK-1* overexpressed NIH 3T3 cells (Fatima et al 2006, Tang et al 2007).

5.3 Molecular characterization of *Timm22* and *JC-1* in human ESCC

Timm22, also known as translocase of inner mitochondrial membrane 22, is a known gene that encodes a protein Tim22 and its functions have been previously investigated. According to the NCBI's GenBank data, the entire sequence of the *Timm22* transcript is 1691bp long. The entire coding sequence of *Timm22* is from 27bp to 611bp and it encodes a 194-amino acid protein – mitochondrial import inner membrane translocase subunit Tim22 [Homo sapiens] (NP_037469). Studies have shown that TIM complex is a 300-kD protein insertion complex that contains 6 subunits including protein Tim22, and it could import polytopic inner membrane proteins with internal targeting signals (Rehling et al 2003) and (Muhlenbein et al 2004). TIM complex is the only essential membrane-integrated subunit of the yeast complex and it mediates the insertion of precursor proteins in a 3-step process (Kovermann et al 2002) and (Rehling et al 2003). Although some studies have been conducted to investigate the functions of Tim22 protein, there is none so far on the correlation between *Timm22* gene and pathogenesis of ESCC and other cancers.

Our present results revealed a remarkable overexpression of *Timm22* in 71.4% (10/14) of ESCC cell lines and 55.2% (16/29) of ESCC patient cases.

Moreover, *Timm22* expression was also observed in NE3 and non-tumor epithelial tissues (89.7%; 26/29). This may indicate that *Timm22* plays its normal functional role in non-tumor esophageal epithelial cells. Our results also disclosed for the first time that overexpression of *Timm22* was found in both ESCC cell lines and patient specimens. Although there were no previous studies on the relationship between *Timm22* and tumor metabolism, studies showed that mitochondria play an important role in altering metabolism in cancer cells (Ramsay et al 2011). Recently, protein translocation into mitochondria and its involvement in apoptosis, ageing, cancer and other cellular processes have attracted much attention. Interactions between components of inner and the outer membrane are essential for several central mitochondrial functions, such as the channeling of metabolites, coordinated fusion and fission of mitochondria and protein transport (Reichert and Neupert 2002). A previous study has shown a high expression level in proliferative cells of an inner mitochondrial nuclear-encoded protein, adenine nucleotide translocator (ANT) 2, which functions as a catalyst in the exchange of mitochondrial ATP with cytosolic ADP, and is known to be directly associated with glycolytic metabolisms and carcinogenesis (Marzo et al 1998). In addition, suppression of ANT2 was reported to effectively induce apoptotic cell death and tumor growth inhibition in breast cancer models *in vitro* and *in vivo* (Jang et al 2008). According to these previous studies on

mitochondrial proteins, the overexpression of *Timm22* may contribute to the metabolic demands of cancer cells as described before. Still, *Timm22* was found underexpressed in a few ESCC tissue specimens, which may be described as other unknown genetic events, e.g. chromosome deletion and/or hypermethylation of the *Timm22* promoter (Kuwano et al 2005). Moreover, immunostaining analysis was carried out in this study to measure the protein expression level of *Timm22* in ESCC cell lines in comparison with NE1 and NE3 cells. Seven ESCC cell lines (HKESC-3, HKESC-4, KYSE 30, KYSE 150, KYSE 450, KYSE 510 and KYSE 520) were stained by mouse polyclonal antibody to human *Timm22* (Abcam). Among the 7 ESCC cell lines, 3 of them (HKESC-3, HKESC-4 and KYSE 30; 42.9%) showed a higher expression level of Timm22 protein than NE1 or NE3 while the other 4 showed less (KYSE 510, KYSE520 and KYSE150) or no (KYSE 450) Timm22 protein expression. This result may indicate that the overexpression of Timm22 protein is only one of the multiple factors that may or may not be acquired by the ESCC cells. Further investigation about the functional role of overexpressed Timm22 protein is required to illustrate its role in molecular carcinogenesis in ESCC.

In the expression study of *Timm22* in ESCC patient specimens, 55.2% (16/29) ESCC patient specimens showed *Timm22* overexpression. By investigating the overexpression pattern of *Timm22* in ESCC patient specimens, the clinicopathological parameters of the patients and *Timm22*

overexpression could be correlated (Table 7).

Among the ESCC patient samples, overexpression of *Timm22* (16 cases) was more common in patients with stage II (50%; 8/16) and stage III (37.5%; 6/16) (Table 7). These *Timm22* overexpressed stage II and III patient samples count for over 85% among all *Timm22* overexpressed samples. Therefore, *Timm22* overexpression may contribute to the tumor progression of ESCC but not to the final stage. To compare the histological subtypes of the ESCC cases with the overexpression pattern of *Timm22*, Table 8 summarizes the results of the expression study in relation to the histological subtypes. The results revealed that more patient cases with *Timm22* overexpression were moderately differentiated carcinoma (9/17; 52.9%) compared to those well differentiated (4/17; 23.5%) and poorly differentiated (4/17; 23.5%) carcinoma.

Although correlation between *Timm22* overexpression and clinicopathological features in ESCC patient cases can be found in the present study, a larger sample size should be included in future studies for establishing more accurate and significant correlation.

Table 7. A summary of results (refer to section 4.3.1, Table 5) showing the distribution of tumor stages of the patient samples with overexpressed *Timm22*:

	Stage I	Stage II	Stage III	Stage IV	Total No.
Total No. of ESCC cases	1	11	14	3	29
No. of cases with Timm22 overexpression	0	8	6	2	16
% of cases with Timm22 overexpression	0% (0/16)	50% (8/16)	37.5% (6/16)	12.5% (2/16)	55.2% (16/29)

Table 8. A summary of results (refer to section 4.3.1, Table 6) showing the distribution of histological subtypes of patient samples with *Timm22* overexpression.

	Well differentiation	Moderately differentiation	Poorly differentiation	Total No.
Total No. of ESCC cases	10	13	6	29
No. of cases with Timm22 overexpression	4	9	3	16
% of cases with Timm22 overexpression	25% (4/16)	56.3% (9/16)	18.8% (3/16)	55.2% (16/29)

It is widely accepted that genomic amplification and overexpression of genes in cancer can contribute to tumor pathogenesis and progression (Nowell 1989), examples were illustrated in the previous discussion (section 5.2). Thus, the high percentage of overexpression of *Timm22* in 10 out of 14 (71.4%) ESCC cell lines observed in the present study may provide some clues about the importance of maintaining *Timm22* overexpression during tumor progression. For example, the existence of the overexpressed *Timm22* can offer growth advantages in 71.4% of the well-established ESCC cell lines. If Timm22 overexpression is eliminated based on the theory of clonal evolution for cancer growth, the overexpression level of Timm22 could not be detected in such a high percentage (Nowell 1986). As a result, further investigation about the role of overexpressed *Timm22* on the tumor transforming capacity was performed and it is discussed in the following sections.

Back in the 1970s, assays for detecting tumor transforming capacity *in vitro* included the measurement of proliferation rate, foci formation assay, and colony formation in soft agar (Adjei 2001, Ito et al 2003, Newbold and Overell 1983, Ponten 1976, Schwarzbach et al 2004). Other reports also indicated that the rate of cell migration in cell cultures may evaluate the tumor transforming capacity relating to tumor metastasis (Cha et al 1996). In the present study, we examined the *in vitro* characteristics of the

transformed phenotypes with overexpressed *Timm22*, such as the proliferation rate, foci formation under confluent growth, anchorage independent growth in soft agar and cell migration. The results indicated that *Timm22* overexpressed non-tumor NIH 3T3 cells gained the transformed phenotypes, i.e. faster growth rate, foci formation ability with loss of contact inhibition, anchorage independent growth ability in soft agar and faster migrating rate. Altogether, these results illustrated that NIH 3T3 cells with *Timm22* overexpression performed like the transformed cells. These phenotypic changes are assumed to be closely related to the process of *in vivo* carcinogenesis.

In the present study, NIH 3T3 non-tumor mouse fibroblast cells were employed for studying the transforming capacity of overexpressed *Timm22*. NIH 3T3 cells were used in this study as they were derived from a well established mouse fibroblast cell line and do not carry any foreign DNA (Todaro and Green 1963). NIH 3T3 cells show properties such as infinite growth, fast growing rate, growth with contact inhibition and tumorigenic potential in nude mice with the overexpression of transfected foreign oncogenes (Bos et al 1987, Ellison and Rubin 1992, Fatima et al 2006, Fujita et al 1984, Kuo et al 2000, Li et al 2005, Moshier et al 1993, Visvanathan et al 1988). These properties have been widely reviewed as ideal for examining the tumor transforming properties of potential oncogenes(Stepanenko and

Kavsan 2012). NE1 and NE3 cells are not ideal for being used in our transformation study although they are non-tumor esophageal epithelial cell lines. This is because NE1 and NE3 cells have been immortalized by human papillomavirus type 16 E6E7 (HPV16 E6/E7), which encodes the viral oncoproteins E6 and E7 (Deng et al 2004, Zhang et al 2006) . Although the viral DNA and oncoproteins are expressed at low level so that they will not directly contribute to the oncogenic transformation in NE1 and NE3 cells, the transfection of foreign target genes with overexpression in NE1 or NE3 cells may induce some unexpected interactions with the viral oncoproteins E6 and E7 and lead to the transforming abilities, which may complicate the interpretation of the results.

Overexpression of *Timm22* mRNA in NIH 3T3 cells increased the cell proliferation rate obviously compared to parental NIH 3T3 and mock vector transfected NIH 3T3 cells in the present study. *Ras* oncogen overexpressed in NIH 3T3 cells also showed increased growth rate, which indicated that both *ras* oncogene and *Timm22* promoted cell proliferation and led to abnormal growth. The *ras* oncogene is located at chromosome 11 and encodes the 21 kDa guanine nucleotide-binding oncoprotein Ras (Adjei 2001). Several reports indicated that about 30% human cancers have *ras* mutant (Adjei 2001, Bos et al 1987, Fujita et al 1984, Visvanathan et al 1988). The Ras protein is employed to regulate cell growth as a key intermediate and can

trigger *ras* pathway of growth factor signaling (Adjei 2001). In the present study, pcDNA3.1-*H-rasV12* was constructed by inserting *H-rasV12* into vector pcDNA3.1 (-) and transfected into parental cells as an oncogenic positive control. In *H-rasV12*, glycine residue was substituted by valine residue at codon 12 in Harvey (H)-Ras (Li et al 2005) to transform *ras* into an oncogenic form. The modification of *ras* gene causes the *H-rasV12* to encode a mutant and active form of Ras protein which loses the intrinsic GTPase (guanosine triphosphate hydrolase enzyme) function. As a result, the protein is constitutively in the active, GTP-bound state and is continuously sending signals for cell growth even in the absence of growth factor and regardless of whether receptors on the cell surface are activated (Lee and Helfman 2004, Rayter et al 1989). Therefore, the pcDNA3.1-*H-rasV12* transfected 3T3/*ras* cells showed a much higher growth rate than the untransfected parental NIH 3T3 and empty mock vector transfected cells. From our results (Figure 23), very similar growth patterns were observed in NIH 3T3 and 3T3/vec cells, which implied that the cloning vector alone did not increase the growth rate of NIH 3T3 cells. In contrast, *Timm22* overexpressed NIH 3T3 cells showed an obvious increase in growth rate which is comparable to the *ras*-overexpressed cells. As a result, the overexpression of *Timm22* did contribute to the growth advantage of non-tumor cells by increasing the growth rate. Thus further investigation

was done to test whether Timm22 showed tumor transforming capacity on non-tumor cells.

The foci formation assay and soft agar assay were conducted to examine the contact inhibited and anchorage-independent growth abilities of cells under confluent condition and in soft agar with or without gene transfection respectively. These are simple experiments very often used to assess the cell transforming capacity with target genes. As reported previously, normal human stromal cells cannot grow on culture plate or flask when the density of the monolayer cells reach confluence, which is also known as contact inhibition (Abercrom.M 1970). According to Madeja and Klein's report, normal human stromal cells such as fibroblasts or epithelial cells have anchorage-dependent property that requires substratum attachment to grow (Madeja and Klein 1990). When fibroblast and epithelial cells have malignant transformation, the ability to proliferate under anchorage-dependent conditions with loss of contact inhibition will emerge as an important sign of cellular transformation and could be detected as foci formation ability and colony formation ability in soft agar (Madeja and Klein 1990, Yang and Vas 1972).

Some previous studies have used NIH 3T3 mouse fibroblast cell line to examine the transforming ability of potential and known oncogenes (Barr and Johnson 2001, Bleiberg et al 1985, Ellison and Rubin 1992, Fatima et al

2006, Fenrick et al 2000, Lorch et al 2001, Xia et al 2000). Our results revealed that (1) NIH 3T3 cells transfected with the positive control *ras* gene and the target *Timm22* gene showed ability to form foci in the *in vitro* confluent culture environment, which revealed the loss of contact inhibition; and (2) ability to generate colonies in soft agar, which demonstrated the anchorage independent growth manner of the transfected cells. In contrast, no colony was observed in the parental NIH 3T3 or mock vector transfected cells as the negative controls.

Furthermore, cell migration rate also reflects cell proliferation and wound healing ability. The migration rate of malignant cells is higher than that of normal cells as the former has metastatic potential (Hattori et al 2009). In our study, *ras* and *Timm22* transfected and overexpressed NIH 3T3 cells could migrate across the scratched-wound faster than the parental and mock vector transfected NIH 3T3 cells. According to all the three results of foci formation, soft agar and migration assays, the gain of anchorage dependent and independent growth and higher migration rate of *Timm22* overepxressed cells implied that *Timm22* overexpression has a close relation with the transformation of normal cells. As a result, it was very crucial to further examine the transforming effect of *Timm22* overexpression by means of *in vivo* tumorigenecity to test whether the cellular transformation would also lead to the malignant growth of the transformed cells.

In vivo tumorigenecity test was conducted using athymic nude mice. This test was widely used previously and was considered as the most reliable indicator of cell malignancy (Fatima et al 2006, Kanda et al 1994, Yang et al 2005). In our study, parental NIH 3T3, *ras*, *Timm22* and mock vector transfected cells were subcutaneously injected separately into each group of three athymic nude mice. The nude mice used in the study had a *nu* mutation that causes defects in developing the cortical epithelium of thymus. The mutation then leads to imperfection of T cell development and finally causes the immunodeficiency of the mice (Janeway et al 2005). Our results showed that injection of *Timm22* transfected NIH 3T3 cells generated undifferentiated sarcoma with cellular fascicles of spindle tumor cells with prominent nuclear pleomorphism in all the tested mice, indicating the tumor transforming capacity of *Timm22* overexpression. Similar results were also shown in *ras* transfected NIH 3T3 cells but no sarcoma was formed in the mice injected with the parental NIH 3T3 or mock vector transfected NIH 3T3 cells. Therefore, summarizing the results of *in vitro* growth rate, foci formation, soft agar and migration assays as well as the *in vivo* tumorigenicity test conducted in this study, they further indicated that overexpression of *Timm22* contributed to malignant transformation of non-tumor NIH 3T3 cells. Together with the observation that 10 out of 14 (71.4%) of ESCC cell lines showed overexpressed Timm22, the overall results

imply the potential impact of *Timm22* overexpression on ESCC tumorigenesis.

Another novel gene located in chromosome 17p13.3, named as *JC-1* in the present study, was characterized for its potential on molecular pathogenesis in ESCC according to its high percentage (78.6%; 11/14) of overexpression in ESCC cell lines. According to the NCBI's GenBank database, the reference sequence of *JC-1* (cDNA) is NM_003585.3, and it was named Homo sapiens double C2-like domains, beta (DOC2B) and the entire sequence is 2022bp long. The entire coding sequence of *JC-1* starts from 151bp and encodes 412 amino acid residues. The *JC-1* protein is named double C2-like domain-containing protein beta [Homo sapiens] and has NCBI reference sequence: NP_003576.2. DOC2B was first reported in 1995. It is one of the two repeated C2-like domains on a brain protein DOC2. DOC2B was found to serve as a Ca^{2+} sensor which interacts with Ca^{2+} and phospholipid in synaptotagmin and Rabphilin-3A, which are implicated in neurotransmitter release (Orita et al 1995). However, the role of *JC-1* in the molecular pathogenesis of ESCC has never been studied before.

As discussed previously, gene overexpression is a common event in ESCC and is usually involved in the mechanisms of molecular pathogenesis in ESCC. Among the ESCC cell lines we examined, 11 out of 14 (78.6%)

showed *JC-1* overexpression comparing with *JC-1* expression level in NE3. The rate of overexpression of *JC-1* was also examined in 31 ESCC specimens, and only one of them (3.2%) showed *JC-1* overexpression. All the other patient specimens showed no changes in the *JC-1* expression level except one specimen showed under-regulated *JC-1* expression level. Moreover, *JC-1* did not show its expression in NE3 and most of the corresponding non-tumor epithelial tissue specimens of ESCC patients in this study, implying the possibility that *JC-1* is not a critical gene for the normal epithelial functions. This result is different from the *Timm22* gene for which the percentage of overexpression of *Timm22* is both high in ESCC cell lines (71.4%) and patient cases (55.2%). The *JC-1* overexpression was observed more often in the well established ESCC cell lines than the patients' non-tumor and tumor samples, and this may imply the outcome of selection of *JC-1* overexpression throughout the course of clonal evolution (over decades) for the *in vitro* growth of the cell lines, and *JC-1* overpression did provide certain growth advantages to the clonal ESCC cell lines. This view is also evidenced by the present study that *JC-1* overexpression could offer growth advantages, e.g. faster growth and migration rates, to the transfected non-tumor cells *in vitro*.

Similar to the investigation on *Timm22*, the same assays for studying the *in vitro* tumor transforming capacity of *JC-1* were employed, and they

were growth proliferation, foci formation, colony formation in soft agar and migration assays. The results implied that *JC-1* overexpressed non-tumor NIH 3T3 cells had higher growth rate, loss of contact inhibition to form foci, anchorage independent growth in soft agar and higher migrating rate. Thus these results suggested that *JC-1* overexpressed NIH 3T3 cells gained the phenotypic changes for the transformed cells, and are closely related to the process of *in vivo* carcinogenesis of ESCC. As described in section 4.3.3, NIH 3T3 with *JC-1* and *ras* overexpression showed increased proliferation rate apparently and it is evidenced that *JC-1* overexpression may contribute to selectable growth advantage of the transformed cells. Moreover, the growth ability with contact inhibition condition was also tested. The results were reported in section 4.3.4. In our study, NIH 3T3 and vector transfected NIH 3T3 cells did not form any foci in culture plates with contact inhibition. In contrast, both *JC-1* and *ras* overexpressed NIH 3T3 cells showed foci with the loss of contact inhibition. According to previous discussion, the ability of the transformed cells to grow with the loss of contact inhibition is one of the characteristic features of transformed cells and was observed in the *JC-1* overexpressed cells. Anchorage-independent growth assay on soft agar was similarly employed in this part of study to assess the anchorage-independent growth ability of *JC-1* and *ras* overexpressed NIH 3T3 cells compared with the parental and mock vector transfected NIH 3T3

cells. From the results (section 4.3.5), parental and vector transfected cells could not form colony in soft agar while *JC-1* and *ras* overexpressed NIH 3T3 cells could form colonies. Thus the findings revealed the increased anchorage-independent growth ability of *JC-1* overexpressed cells. Together with the results of the migration assay that the *JC-1* and *ras* overexpressed NIH 3T3 cells showed a faster migration rate across the scratched-wound than the parental and vector transfected NIH 3T3 cells (section 4.3.6), *JC-1* overexpressed cells acquired the critical phenotypic changes which are commonly found in malignant cells. Thus this is the first report about *JC-1* overexpression in association with the transformation of normal cells. Further investigation also confirmed the *in vivo* tumor transforming ability of *JC-1* overexpressed NIH- 3T3 cells in nude mice test (section 4.3.7). The overall results of *JC-1* study identified for the first time the oncogenic potential of *JC-1* when it is overexpressed in non-tumor NIH 3T3 cells.

5.4 Overall discussion

ESCC is an aggressive malignant cancer and it is the 8th most common cancer deaths for both sexes in Hong Kong (Hong Kong Cancer Stat 2009). Its 5-year survival rate remains less than 10% with multimodality treatments for decades (Kwong et al 2004). It is believed that the findings of novel molecular targets will be beneficial to the understanding of the molecular pathogenic mechanisms of the disease and to the discovery of novel therapeutic targets which may improve the survival rate of ESCC in the long run.

Homogeneously staining regions (HSRs) are chromosomal segments commonly found in cancer cells appeared with greatly lengthened and expanded morphology and they are usually associated with gene amplification and/or overexpression (Biedler and Spengler 1976). In the present study, a HSR was first identified in SLMT-1, an ESCC cell line of Hong Kong Chinese origin. Our overall findings led to the discovery of the oncogenic properties of *Timm22* and *JC-1*. *Timm22* overexpression is commonly found in ESCC patient samples and opens a chapter for the future investigation about the functional roles of overexpressed *Timm22* in the transformation processes of non-tumor cells. Further studies can include microarray analyses to identify downstream regulatory targets with Timm22 overexpression or knock-down studies. Then functional studies of Timm22 in

transformed cells can also be followed up accordingly by microarray assays.

It has been indicated that the tumor progression of gastrointestinal (GI) cancer requires multiple molecular changes including various oncogenes, tumor suppressor genes and cell-cycle regulatory genes (Schuuring 1995, Tahara 1995). Our group also investigated some novel genes that may be related to ESCC pathogenesis in regional amplification of 5p contig, *JS-1* (Fatima et al 2006) and *JK-1* (Tang et al 2007), as well as tumor transforming gene *GAEC1* at 7q22 (Law et al 2007). *JS-1, JK-1* and *GAEC1* showed overexpression in most ESCC cell lines and they demonstrated the characteristics of transforming capacities in normal cells. Other recent efforts on ESCC related gene amplification included genes *GASC1* (Yang et al 2000), *EC97* (Lu et al 2003) and *mimitin* (Tsuneoka et al 2005). Thus the findings of the present study contribute more to the current understanding about the molecular pathogenesis of ESCC. We believed that *Timm22* overexpression in ESCC may contribute to the metabolic demands of cancer cells since the protein complex TIM22 which involved Timm22 protein is responsible for transporting proteins across the inter membrane space of mitochondria. Further in-depth investigation about the role of *Timm22* overexpression and mitochondrial functions is another direction of research initiating by the present study.

Chapter 6
Summary and conclusions

In the present study, the identification and characterization of 17p HSR led to the novel study of *Timm22* and *JC-1* in ESCC and the results are summarized as follows:

(I) The origin of the HSR identified in SLMT-1 was located in chromosome 17p and it is between the positions of the BAC clones RP11-1260E13 and RP11-216P6.

(II) *Timm22* and *JC-1* were overexpressed in 71.4% (10/14) and 78.6% (11/14) of ESCC cell lines respectively. *Timm22* overexpression is most commonly found in stage II and stage III ESCC patient samples (50% and 37.5% respectively). *JC-1* ovexpression is not commonly found in the ESCC patient samples.

(III) Both *Timm22* and *JC-1* overexpressed NIH 3T3 cells acquired *in vitro* and *in vivo* tumor transforming features, including higher proliferation rate, loss of contact inhibition, anchorage independent growth, higher migration rate and formation of subcutaneous tumors in nude mice.

Two newly involved genes *Timm22* and *JC-1* in ESCC were identified in

the present study and they showed tumor transforming capacities in normal cells. Therefore, *Timm22* and *JC-1* may play an important role in the molecular pathogenesis of ESCC and deserve further investigations to understand more about their molecular mechanisms of pathogensis in ESCC and possibly in other cancers in future.

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Appendices

Appendices A: Information for *JC-1*

(I) General Information for *JC-1*

Accession no.	NM_003585
Source	Homo sapiens (human)
Definition	Homo sapiens double C2-like domains, beta (DOC2B), mRNA
Location	Chromosome 17p13.3
Entire length of gene	2022bp
Coding sequence (CDS)	151-1389
Predicted protein	412 amino acids
Function	Unknown

(II) Coding sequence of *JC-1*

“ATGACCCTCCGGCGGCGCGGGGAGAAGGCGACCATCAGCATCCAGGAGCATATG
GCCATCGACGTGTGCCCCGGCCCCATCCGTCCCATCAAGCAGATCTCCGACTACTTC
CCCCGCTTCCCGCGGGGCCTGCCCCCGGACGCCGGGCCCCGAGCCGCTGCACCCC
CGGACGCCCCCGCGCGCCCGGCTGTGGCCGGTGCCGGCCGCCGCAGCCCCTCCGA
CGGCGCCCGCGAGGACGACGAGGATGTGGACCAGCTCTTCGGAGCCTACGGCTCC
AGCCCGGGCCCCAGCCCGGGTCCCAGCCCCGCGCGGCCGCCAGCCAAGCCGCCG
GAGGACGAGCCGGACGCCGACGGCTACGAGTCGGACGACTGCACTGCCCTGGGC
ACGCTGGACTTCAGCCTGCTGTATGACCAGGAGAACAACGCCCTCCACTGCACCAT

CACCAAGGCCAAGGGCCTGAAGCCAATGGACCACAATGGGCTGGCAGACCCCTAC
GTCAAGCTGCACCTGCTGCCAGGAGCCAGTAAGGCAAATAAGCTCAGAACAAAAA
CTCTCCGTAACACTCTGAACCCCACATGGAACGAGACCCTCACTTACTACGGGATCA
CAGATGAAGACATGATCCGCAAGACCCTGCGGATCTCTGTGTGTGACGAGGACAAA
TTCCGGCACAATGAGTTCATCGGGGAGACACGTGTGCCCCTGAAGAAGCTGAAAC
CCAACCACACCAAGACCTTCAGCATCTGCCTGGAGAAGCAGCTGCCGGTGGACAA
GACCGAAGACAAGTCCCTGGAGGAGCGGGGCCGCATCCTCATCTCGCTCAAGTAC
AGCTCACAGAAGCAAGGCCTGCTGGTAGGCATCGTGCGGTGCGCCCACCTGGCCG
CCATGGACGCCAACGGCTACTCGGACCCCTACGTGAAAACATACCTGAGGCCAGAT
GTGGACAAGAAATCCAAACATAAGACAGCGGTGAAGAAAAAAACCCTGAACCCGG
AGTTTAATGAGGAGTTCTGTTACGAGATCAAGCATGGGGACCTGGCCAAGAAGTCC
CTGGAGGTCACCGTTTGGGATTACGACATTGGAAAATCCAACGATTTCATTGGTGGT
GTGGTTCTGGGCATCCACGCCAAGGGGGAGCGCCTGAAGCACTGGTTTGACTGCC
TGAAGAACAAGGACAAGCGCATCGAGCGCTGGCACACGCTCACCAGCGAGCTCCC
AGGGGCTGTGCTCAGCGACTGA”

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

“MTLRRRGEKATISIQEHMAIDVCPGPIRPIKQISDYFPRFPRGLPPDAGPRAAAPPDAP
ARPAVAGAGRRSPSDGAREDDEDVDQLFGAYGSSPGPSPGPSPARPPAKPPEDEPDA
DGYESDDCTALGTLDFSLLYDQENNALHCTITKAKGLKPMDHNGLADPYVKLHLLPGA
SKANKLRTKTLRNTLNPTWNETLTYYGITDEDMIRKTLRISVCDEDKFRHNEFIGETRVP
LKKLKPNHTKTFSICLEKQLPVDKTEDKSLEERGRILISLKYSSQKQGLLVGIVRCAHLAA
MDANGYSDPYVKTYLRPDVDKKSKHKTAVKKKTLNPEFNEEFCYEIKHGDLAKKSLEVT
VWDYDIGKSNDFIGGVVLGIHAKGERLKHWFDCLKNKDKRIERWHTLTSELPGAVLSD
”

Appendices B: Information for *Timm22*

(I) General Information for *Timm22*

Accession no.	NM_013337
Source	Homo sapiens (human)
Definition	Homo sapiens translocase of inner mitochondrial membrane 22 homolog (yeast) (TIMM22), nuclear gene encoding mitochondrial protein, mRNA
Location	Chromosome 17p13.3
Entire length of gene	1691bp
Coding sequence (CDS)	27-611
Protein	194 amino acids
Function	import polytopic inner membrane proteins with internal targeting signals

(II) Coding sequence of *Timm22*

“ATGGCGGCGGCCGCCCCCAATGCCGGAGGCTCGGCCCCTGAGACAGCGGGTTCC
GCCGAAGCTCCGCTGCAGTACAGCCTGCTCCTGCAGTACCTGGTGGGTGACAAGCG
TCAGCCCCGGCTCCTGGAGCCTGGGAGCCTGGGCGGGATCCCAAGTCCAGCCAAG
AGTGAGGAGCAGAAGATGATCGAGAAGGCGATGGAAAGCTGCGCTTTCAAGGCT
GCGCTGGCCTGCGTGGGAGGATTTGTCTTAGGAGGTGCATTTGGGGTGTTTACCGC
TGGCATCGATACCAACGTGGGCTTTGACCCTAAGGATCCTTACCGTACACCGACTGC
AAAAGAAGTGCTGAAAGACATGGGGCAGAGAGGAATGTCCTATGCCAAAAATTTC
GCCATTGTGGGAGCCATGTTTTCTTGTACTGAGTGTTTGATAGAATCTTACCGGGGA
ACATCAGACTGGAAGAACAGTGTCATCAGTGGCTGCATCACGGGAGGAGCTATTGG

TTTCAGAGCTGGCTTAAAGGCTGGGGCCATTGGTTGTGGAGGTTTTGCTGCTTTCT
CTGCTGCGATTGATTATTACCTCCGGTGA”

(III) Protein sequence after *Timm22* translation

“MAAAAPNAGGSAPETAGSAEAPLQYSLLLQYLVGDKRQPRLLEPGSLGGIPSPAKSEE
QKMIEKAMESCAFKAALACVGGFVLGGAFGVFTAGIDTNVGFDPKDPYRTPTAKEVL
KDMGQRGMSYAKNFAIVGAMFSCTECLIESYRGTSDWKNSVISGCITGGAIGFRAGLK
AGAIGCGGFAAFSAAIDYYLR"

Appendices C: Websites

National Cancer Institute – SEER Training Modules – Anatomy – Head & Neck Overview
http://training.seer.cancer.gov/head-neck/anatomy/overview.html

The Encyclopedia of Science – Digestive System
http://www.daviddarling.info/encyclopedia/D/digestive_system.html

National Cancer Institute – what is cancer (02/06/2012 updated)
http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer

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http://en.wikipedia.org/wiki/Cancer_staging

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http://www.medinfographics.com/cancer-statistics/esophageal-cancer/esophageal-cancer-survival-by-year-of-diagnosis/

Sanger Institute, UK
http://www.sanger.ac.uk/research/projects/molecularcytogenetics/

RPCI-98 library
http://www.chori.org/bacpac/

Ensembl human genomic data
http://www.ensembl.org/Homo_sapiens/Cytoview

Ensembl human genomic data release 46
http://www.ensembl.org

National Center for Biotechnology Information
http://www.ncbi.nlm.nih.gov/

Primer 3 program
http://frodo.wi.mit.edu/primer3/

CGH database
http://www.cghtmd.jp/CGHDatabase/about/introduction_e.htm)