

**IDENTIFYING POTENTIAL BIOMARKERS IN THE  
SECRETOME OF ORAL CANCER CELL LINES BY  
COMPARATIVE PROTEOMIC ANALYSIS**

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## ABSTRACT

Early detection cancer associated biomarker is believed to improve the prognosis outcome of oral cancer by allowing for early treatment. However, discovery of potential oral cancer biomarkers in sera is like looking for a needle in haystack due to the large dynamic range in the concentration of serum protein. Therefore, a study secretome of established cell lines is proposed to bypass such obstacle to discover those secreted biomarkers from tumor mass. Most oral cancer biomarkers discovery researches on secretome of cell lines lacks normal cell lines as control. In this study, several normal primary cultures (316N, 317N, 322N, 326N) were successfully established without hTERT immortalization. By comparison the proteomes of cancer cell lines (48T, 153T, H400) and normal primary cultures using 2D gel electrophoresis, 31 protein identities were recognized have changed in abundance in the cancer cell lines secretome. Bioinformatic analysis of these proteins showed that all identified proteins were possibly secreted in either a signal dependant pathway or golgi independent pathways. The analysis also demonstrated that their expression dynamics were relevant to cancer progression. A concomitant qPCR validation of selected proteins transcript levels demonstrated that metalloproteinase VII (MMP-7), heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2/B1), peroxiredoxin-1 (PRDX1), tissue inhibitor metalloproteinase-1 (TIMP1), laminin beta 3 (LAMB3), interleukin-1 receptor antagonist (IL1RN), calcium binding protein S100-A8 (S100A8), and Secreted protein, acidic, cysteine-rich (SPARC) were significantly differential expressed ( $p=0.05$ ) and worthwhile for further investigation. With further studies, these proteins may be developed into potential diagnostic and prognostic marker candidates for oral cancer.

## ABSTRAK

Biopenanda pengesanan barah awal dapat memanjangkan hayat pesakit barah mulut kerana rawatan pada peringkat awal barah adalah lebih berkesan. Walaupun begitu, penemuan biopenanda barah mulut yang berada di dalam serum adalah amat mencabar kerana protein serum wujud dalam julat dinamik yang amat besar. Untuk mengatasi masalah tersebut, pengajian rembesan protein sel selanjara dicadangkan sebagai pendekatan alternatif bagi meningkatkan kebarangkalian menemui biopenanda yang dirembeskan daripada barah mulut. Walaubagaimanapun, kekurangan sel selanjara yang sebagai kawalan dihadapi oleh kebanyakan penyelidikan. Oleh itu, beberapa sel selanjara normal (316N, 317N, 322N, 326N) dan berjangka hayat pendek tanpa “hTERT immortalization” telah berjaya ditumbuhkan dalam kajian ini. Berdasarkan perbandingan gambar gel 2D rembesan protein antara sel selanjara barah mulut (48T, 153T, H400) dan sel selanjara normal, sebanyak 31 jenis protein telah ditemukan. Kuantiti protein sel barah tersebut adalah berbeza daripada sel normal secara ketara. Berpandukan kepada analisis bioinformatik, semua protein-protein yang dikenalpastikan berkemungkinan dirembeskan mengikut laluan yang berdasarkan isyarat peptida ataupun laluan lain yang baru. Analisis tersebut juga menunjukkan perubahan ekspresi protein-protein tersebut adalah berkaitan dengan perkembangan barah mulut. Pengesanan ekspresi gen telah dilakukan menggunakan “real-time PCR” dan pengesanan tersebut telah menunjukkan bahawa “metalloproteinase VII” (MMP-7), “heterogeneous nuclear ribonucleoprotein A2/B1” (HNRNPA2/B1), “peroxiredoxin-1” (PRDX1), “tissue inhibitor metalloproteinase-1” (TIMP-1), “laminin beta 3” (LAMB3), “interleukin-1 receptor antagonist” (IL1RN), “calcium binding protein S100-A8” (S100A8), dan “Secreted protein, acidic, cysteine-rich” (SPARC) adalah berbeza kuantiti antara normal dan barah secara ketara ( $p = 0.05$ ) dan bernilai untuk siasatan selanjutnya. Dengan kajian yang lebih lanjut, protein-protein ini berpotensi untuk

diketengahkan sebagai calon protein biopenanda diagnostik dan prognostik bagi kanker mulut.

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## LIST OF ABBREVIATIONS

### A

ACN	– Acetonitrile
ADAMs	– A disintegrin and metalloproteases
AML	– Acute myeloid leukemia
ANOVA	– Analysis of variance
ANP32B	– Acidic nuclear phosphoprotein 32 family member B
AP-1	– Activator protein 1
Apaf1	– Apoptotic protease activating factor 1
APS	– Ammonium persulfate

### B

Bcl-2	– B-cell lymphoma 2
BSA	– Bovine serum albumin

### C

°C	– Degree celcius
%C	– The weight percentage of crosslinker
4-CHCA	– $\alpha$ -cyano-4-hydroxycinnamic acid
Ca <sup>2+</sup>	– calcium ion charged +2
CALML3	– Calmodulin-like protein 3
CD95R	– Cluster of differentiation 95 receptor
CDH2	– Cadherin-2 precursor; N-cadherin
cDNA	– Complementary deoxyribonucleic acid
CFL1	– Cofilin-1

CID	– Collision induced dissociation
cm	– Centimeter
cm <sup>2</sup>	– Centimeter cube
CO <sub>2</sub>	– Carbon dioxide
CST6	– Cystatin-M
Ct	– Cycle threshold
CTSD	– Cathepsin D
CypA	– Cyclophilin A (gene name: PPIA)

## **D**

ddH <sub>2</sub> O	– Double distilled water
2D	– Two dimensional
2DE	– Two dimensional gel electrophoresis
DIGE	– Difference gel electrophoresis
DMEM-F12	– Dulbecco's modified eagle medium-F12
DMSO	– Dimethyl sulfoxide
DNA	– Deoxyribonucleotide acid
DTT	– Dithiothreitol

## **E**

ECM	– Extracellular matrix
EDTA	– Ethylenediaminetetraacetic acid
EEFID	– Elongation Factor 1-delta
EF-1	– Elongation Factor 1
EGF	– Epidermal growth factor
EGFR	– Epidermal growth factor receptor



ELISA	– Enzyme-linked immunosorbent assay
EMT	– Epithelial-mesenchymal transition
EPF	– Early pregnancy factor
ERK1/2	– Extracellular signal-regulated kinase 1/2

## **F**

FAD	– Flavin adenine dinucleotide
FASL	– Fas ligand
FasR	– Fas receptor
FBS	– Fetal bovine serum
FDR	– False discovery rate
FGF	– Fibroblast growth factor
Flt1	– Vascular endothelial growth factor receptor 1
FRPs	– Follistatin related proteins
FSTL1	– Follistatin like-1

## **G**

g	–Standard gravity
G1	– Gap 1
Gal-7	– Galectin-7 (protein name)
GGCT	– Gamma glutamylcyclotransferase
GNB2L1	– Guanine nucleotide-binding protein subunit beta 2-like 1
GRD	– Glycine rich domain
GSTO1	– Glutathione transferase omega-1

## **H**

h	– Hour
HCl	– Hydrochloric acid
HDGF	– Hepatoma derived growth factor
hMSH2	– Human mutS homolog 2
HNRNP A2/B1	– Heterogeneous nuclear ribonucleoproteins A2/B1
HNSCC	– Head and neck squamous cell carcinomas
HPV	– Human papilloma virus
Hsp10	– Heat shock protein 10 (protein name)
HSPE1	– Heat shock protein 10 (gene name)

## **I**

IAA	– Iodoacetamide
IL-1	– Interleukin-1
IL1RN	– Interleukin-1 receptor antagonist
IL-6	– Interleukin-6
IPG	– Immobilized pH gradient

## **K**

kDa	– Kilo-Dalton
Ki-67	– cancer antigen Ki-67
KSFM	– Keratinocyte serum free media
kVh	– Kilo-volt hour

## **L**

μL	– Microliter
----	--------------

LAMA3	– Laminin subunit alpha-3
LAMB3	– Laminin subunit beta-3
LEI	– Leukocyte elastase inhibitor (protein name; same as SERPINB1)
LGALS7	– Galectin-7 (gene name)
<b><u>M</u></b>	
M	– Molarity
MALDI	– Matrix-assisted laser desorption/ionization
MALDI-TOF/TOF	– Matrix-assisted laser desorption/ionization-time of flight/time of flight
Mcl-1	– Myeloid cell leukemia sequence 1
MDM-2	– Mouse double minute 2 homolog
MHC	– Major histocompatibility complex
min	– Minutes
mL	– Milliliter
mM	– Millimolarity
mm <sup>2</sup>	– Millimeter square
MMP7	– Matrix metalloproteinase-7
MMPs	– Matrix metalloproteinases
mRNA	– Messenger ribonucleic acid
MS/MS	– Tandem mass spectrometry
MTT	– 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVBs	– Multivesicular bodies

## **N**

- NADH – Nicotinamide adenine dinucleotide
- NF- $\kappa$ B – Nuclear factor-Kappa B
- nm – Nanometer
- NSCLC – Non-small cell lung cancer

## **O**

- OSCC – Oral squamous cell carcinoma

## **P**

- p27 – gene encoded cyclin-dependent kinase inhibitor 1B
- p38 MAPK – p38 mitogen-activated protein kinases
- p53 – Tumor protein 53
- PBS – Phosphate buffer saline
- PCNA – Proliferating cell nuclear antigen
- PCR – Polymerase chain reaction
- PDGF – Platelet-derived growth factor
- pI – Isoelectric point
- PI3-K – Phosphatidylinositol 3-kinase
- PP2A – Protein phosphatase 2A
- PPIA – Peptidylprolyl isomerase A (protein name: CypA)

## **Q**

- qPCR – Quantitative polymerase chain reaction

## **R**

- RB – Retinoblastoma
- RCN1 – Reticulocalbin-1
- RNA – Ribonucleic acid
- RNAi – RNA interference
- RQ – Relative quantity

## **S**

- S – Synthesis
- S100A8 – S100 calcium binding protein A8
- SCC – Squamous cell carcinoma
- SCID – Severe combined immunodeficiency
- SDS – Sodium dodecyl sulphate
- SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
- SERPINB1 – Clade B serpin (gene name of LEI)
- SET – Protein SET
- shRNA – Small hairpin RNA
- siRNA – Small interfering RNA
- SPARC – Secreted protein acidic and rich in cysteine
- SSBP1 – Single-stranded DNA-binding protein

## **T**

- %T – Total monomer concentration
- TEMED – Tetramethylethylenediamine
- TFA – Tetrafluoro acetic acid
- TGF- $\beta$  – Transforming growth factor-beta

TIMP1	– Tissue inhibitor metalloproteinase 1
TIMP2	– Tissue inhibitor metalloproteinase 2
TLR4-	– Toll-like receptor 4
TNF	– Tumor necrosis factor
TNF- $\alpha$	– Tumor necrosis factor-alpha
TP53	– Tumor protein 53

## U

UBB	– Ubiquitin
UV	– Ultraviolet

## V

V	– Voltage
v/v	– Volume/volume
VEGF	– Vascular endothelial growth factor

## W

W	– Watt
w/v	– Weight/volume

## Y

YAG	– Yttrium aluminium garnet
-----	----------------------------

**CHAPTER 1**

**INTRODUCTION**

**&**

**OBJECTIVES**

## 1.1 Introduction:

Oral cancer is the 16<sup>th</sup> most common cancer worldwide. The age-standardized incidence and mortality in Malaysia are approximately 3.5 and 1.5 per 100,000 people annually (Ferlay *et al.* 2010). No significant improvement of incidence and mortality of oral cancer has been observed in Malaysia over several decades since a report by Hirayama in 1966 (Hirayama 1966). Inadequate public awareness of enumerated risk factors of oral cancer in Malaysia was believed to account for such dismal statistic. Exposure to risk factors such as betel quid chewing, tobacco smoking and alcohol consumption may increase the odds of oral cancer development in an individual.

The five-year survival rate of oral cancer is particularly low at stage III and IV due to regional lymph node metastasis, local recurrence and secondary cancer occurrence which render current therapies ineffective. Unfortunately, most oral cancer patients in Malaysia were diagnosed at their advanced stage (Greene *et al.* 2002). In contrast, patients diagnosed at stage I and II have higher survival rate. Thus, an alternate strategy to improve prognosis of oral cancer is early detection (Greene *et al.* 2002; Etzioni *et al.* 2003).

In the context of early-detection of oral cancer, precancerous lesions in oral cavity namely leukoplakia and erythroplakia are ubiquitously used by dental professions as early markers (Kramer *et al.* 1978). Visual examination of the oral cavity has to be performed regularly for early-detection. However, it is impractical and inefficient to regularly screen a large group of people. Unaided oral examination also has relatively lower sensitivity and specificity compared to other visually accessible cancers such as skin melanoma. Low sensitivity and specificity of oral cancer early-detection may lead to overdiagnosis and more false-negative findings (Lingen *et al.* 2008). In addition,



mistakes in diagnosis, delays in obtaining biopsy report, and asymptomatic of precancerous lesions may cause delays in administering therapies. This may provide more time for transformation of indolent tumor into lethal malignant cancer (Khoo *et al.* 1996). As a result, there is a pressing need for identifying new diagnostic aids such as serological biomarkers to assist dental practitioners in making definitive diagnosis and screening efficiently in a non-invasive way.

Discovery of cancer serological biomarkers are being extensively performed by researchers worldwide due to its accessibility and direct reflection of real-time body status. Cancer serological biomarker identification works are initiated based on a paradigm that these aberrantly expressed protein biomarkers are secreted and shed or leaked from tumor masses into bodily fluids or the blood stream (Lescuyer *et al.* 2007). This family of proteins is termed as the cancer secretome. This subproteom encompasses extracellular proteins implicated in anti-apoptosis, adhesion and deadhesion, migration, extracellular matrix (ECM) remodeling, angiogenesis, immune escape and limitless replication which confer plasticity and immortality to tumor cells (Xue *et al.* 2008). As a result, studying the cancer secretome in serum or plasma should improve the likelihood to discover cancer associated biomarkers candidates.

Serum or plasma is a good source of secreted proteins for proteomics study due to its accessibility and homogeneity of protein composition. However, serum proteomics is made difficult by the large dynamic range of serum protein concentration. In other words, secreted proteins of cancerous tissues which are low in quantity will be masked by abundant proteins derived from other tissue sources. Such “masking effect” render cancer secreted proteins undetectable (Xue *et al.* 2008). In order to bypass such

issues, studying secretome derived from established cancer cell lines is an alternative strategy.

Cancer cell lines originated from different anatomic site have distinct molecular expression pattern (Severino *et al.* 2008). Thus, cancer cell lines and their normal counterparts studied by gel-based proteomics in this report were derived from the same anatomic site. This selection constraint may improve result consistency and specificity of biomarker candidates by minimizing molecular heterogeneity of anatomic sub site. Gel-based proteomics used in this project involved two-dimensional gel electrophoresis coupled with tandem mass spectrometry. Potential biomarker candidates identified in this study will have to be further validated for their accuracy as diagnostic or prognostic markers. By understanding their roles in carcinogenesis may help us to gain more insight in oral cancer development. It is also important that they may be potential therapeutic targets for oral cancer drug discoveries.

## 1.2 Objectives

The objectives of this project are:

- Identifying differentially expressed proteins in the secretome of cultured oral cancer cell lines.
- Validating their expression dynamics by real-time PCR.

Those differential expressed proteins are potential to be the oral cancer biomarker candidates or therapeutic targets. The study of their expression dynamics may eventually improve our understanding of the oral cancer progression.

## **CHAPTER 2**

### **LITERATURE REVIEWS**

## 2.1 Epidemiology of Oral Cancer in Malaysia

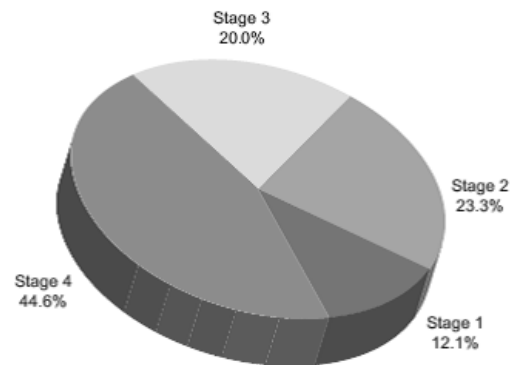
There are 3.5 oral cancer patients for every 100,000 Malaysians in a year and approximately 1.5 oral cancer patients are deceased within 100,000 Malaysian annually (Ferlay *et al.* 2010). Retrospectively, a study conducted by Hirayama *et al* reported that 3.1 new cases were estimated per 100,000 persons in Malaysia for the year 1963 (Hirayama 1966). Obviously, a downtrend of oral cancer incidence in Malaysia is still unseen in over a half century even though numerous researches on oral cancer have been carried out worldwide.

Between the years from 2003 to 2005, the National Cancer Registry, Malaysia documented age-standardized incidence of mouth cancer for Indian male and female at Peninsular Malaysia were 4.4 and 14.5 respectively. It is followed by Chinese male and female, 1.3 and 0.6 respectively. Malay male and female had the lowest age-standardized incidence rates that were 0.7 and 0.8 respectively. Furthermore, Indian male and female had the highest age-standardized incidences of tongue cancer that were 6.0 and 4.9 respectively. Chinese and Malays were ranked second and third in the age-standardized rate of tongue cancer (Lim *et al.* 2008).

For the year 2006, Malaysian Cancer Statistics demonstrated that age-standardized rate of mouth cancer in Indian male and female groups were still lingering higher at 2.4 and 11.5 respectively. Whereas, age-standardized incidence of mouth cancer in Malay and Chinese ethnic groups are remain stable as in the previous years. The estimated age-standardized incidence of tongue cancer for both sexes was 1.3 cases per 100,000 persons. The Indian ethnic group was still ranked top among other races in the incidence of tongue cancer (Ariffin *et al.* 2010).

In the year 2007, the Malaysian Cancer Statistics categorized lip, tongue and mouth cancer under same category, namely oral cancer. Its statistical results indicated the Indian male and female ethnic groups were predominantly inflicted by oral cancer where their age-standardized incidence rates were 3.8 and 10.2 respectively. The National Cancer Registry report 2007 also reported the distribution of diagnosed oral cancer patients based on their tumor staging. It demonstrated that the number of stage IV oral cancer patients accounted for 44.6% of total oral cancer patients in Malaysia, followed by stage II, III, and I, as shown in Figure 2.1 (Ariffin and Saleha 2011).

Collectively, oral cancer incidence ratio of male and female groups is approximately 1:1. However, it has to be noted that the Indian female group has a higher incidence rate of mouth cancer than the male group. This may be associated with Indian females more frequently indulging in betel quid chewing (Tan *et al.* 2000). All these statistics and studies substantiate an apparent association between ethnic background and prevalence of oral cancer in Malaysia (Ramanathan and Lakshimi 1976; Ng *et al.* 1985).



**Figure 2.1:** Distribution of stage at diagnosis of oral cancer at Malaysia 2007. **Source of reference:** Ariffin, O. Z., & Saleha, I. T. N. (2011) National Cancer Registry Report: Malaysia Cancer Statistics - Data and Figure 2007. Ministry of Health, Malaysia.

## 2.2 Etiology of Oral Cancer

With the Indians and the indigenous people of Sabah and Sarawak being the most vulnerable group, it is likely that the variation in incidence rates among populations is due to differences in predisposing factors such as aging, low intake of fruit and vegetable, tobacco smoking, alcohol consumption, and quid chewing (R.B.Zain and N.Ghazali 2001).

A close association between betel quid chewing and oral cancer has been recognized for many years. A cohort study in Taiwan by Shiu M. N. *et al* demonstrated that betel quid chewers were 26 times more likely to develop leukoplakia – a precancerous lesion compared to control subjects. Their report also indicated that betel quid chewers has a higher likelihood of malignancy transformation compared to individuals exposed to other etiologic factors (Shiu *et al.* 2000). In a case-control study conducted by Hirayama in Kuala Lumpur for the year 1963, it was shown that heavy quid chewers in the cancer group were more than the control group. This result

suggested that the heavy quid chewers have a higher risk of developing oral cancer (Hirayama 1966).

In addition, two retrospective studies of case series with a total 1643 cases over two time periods 1967 – 1972 and 1978 – 1984 at the Institute of Medical Research (IMR), Malaysia further supported the association between prevalence of oral cancer and betel-quid chewing. It was also observed that the most prevalent site of oral cancer was the buccal mucosa for quid chewers (R.B.Zain and N.Ghazali 2001). A cohort study by Tan B. S. at six Malaysian estates showed that the quid chewing habit is mainly initiated by family and friends. It also reported that mixing tobacco and lime in betel quid are associated with higher occurrences of precancerous lesions (Tan *et al.* 2000). With respect to carcinogens, the basic ingredient of betel-tobacco quid areca nut, betel-leaf, slaked lime and tobacco has been implicated as the causative agents in oral carcinogenesis. The alkaloid and polyphenolic constituents in the areca nut and betel leaf are analogous to DNA alkylating agents. Nitrosamine, a notorious carcinogen can be produced from the reaction of betel arecoline, nitrites, and thiocyanate *in vivo*. Slaked limes in the quid aid in oral carcinogenesis by promoting the release of free alkaloids from the betel quid (Awang 1988).

The link between tobacco consumption and oral cancer has been firmly established for many decades with substantial epidemiological studies (R.B.Zain 2001). Tobacco consumption is widely practiced among Malaysian people. In the years 1993 - 1994, a nationwide cancer survey reported that a prevalence of 23.2 % of current smokers who are aged 25 years old and above. While, a prevalence of 24.8% was reported for current smokers aged 18 and above for the year 1996 (R.B.Zain and N.Ghazali 2001). The major carcinogenic compounds in cigarette smoke are



nitrosamine and benzopyrene which are implicated in DNA adduct formation in a cell (Silverman 1998).

Alcohol consumption is another etiological factor for oral cancer. However, the prevalence of alcohol consumption in Malaysians is relatively low compared to other risk factors due to ethnic and religious sensitivities. A survey conducted in 1996 among non-Muslims showed a 23% prevalence of alcohol drinkers in Malaysia. The Indians accounted for the large proportion of alcohol drinkers in Malaysia (approximately 13%), followed by the indigenous people of Sabah and Sarawak; (10%) and the Chinese; (7.8%) (R.B.Zain and N.Ghazali 2001). The synergistic effect of alcohol consumption and tobacco smoking has been demonstrated in many studies from different countries (R.B.Zain 2001). It potentiates the carcinogenic effect of tobacco at every level of tobacco use. In this respect, alcohol may render the oral mucosa more vulnerable to carcinogens (Silverman 1998). It is further supported by a study using an in-vitro model of porcine oral mucosa to demonstrate that ethanol enhanced the penetration of N-nitrosornoncotine (a tobacco associated carcinogen) across oral mucosa (Du *et al.* 2000).

Environmental factors are also risk factors in developing oral cancer. These include humanpapilloma virus (HPV) infection, *Candida* species fungal infection, UV exposure, poor oral hygiene, medical immunosuppression, and syphilis (Kumar and R.B.Zain 2004). Notably, HPV infection has been associated with several anogenital and non-anogenital cancers (such as uterine cervix cancer, head and neck squamous cell carcinomas). Their cancer inducing ability is largely attributed to their E6/E7 oncoproteins which are able to disrupt retinoblastoma protein and degrade p53 leading to uncontrollable cell growth. Among the hundreds of types of HPV, HPV-16 is the

most frequently found in the majority of OSCC followed by HPV-18 (Munoz *et al.* 2006). Studies in Malaysia demonstrated that HPV infection is significantly associated with oral cancer and there is a low risk of vertical transmission from women with cervical cancer to their children (Saini *et al.* 2010; Saini *et al.* 2011). Environmental risk factors of oral cancer can be avoided by understanding the cancer associated virus transmission routes and maintaining personal hygiene. As a result, frequent exposure to such risk factors may contribute to a higher chance for oral cancer developing in an individual.

### **2.3 Oral cancer and Prognosis**

Oral cancer is clinically defined as malignant lesion within oral cavity. The mucosal surface of the oral cavity is divided into eight subsites that are the mucosal lip, buccal mucosa, lower alveolar ridge, upper alveolar ridge, retromolar gingival, floor of the mouth, hard palate, and anterior two-third of the tongue. Most cancerous oral cells originate from the oral squamous epithelium cell which is the primary surface structure of the lips and mucous membrane of the oral cavity (Sugerman and Savage 1999). Furthermore, OSCC has been histologically characterized as irregular nests, columns or malignant epithelial cells which was infiltrating subepithelially to surrounding tissue such as the subepithelial connective tissue, adipose tissue, muscle, vascular spaces, bone or neural bundle (R.B.Zain and N.Ghazali 2001). Abnormalities of oral cancerous cells are the result of several consecutive genetic mutations.

The genetic progression model of epithelial carcinogenesis is widely adopted to demonstrate oral carcinogenesis. It begins with a persistent and stepwise accumulation of mutation inside the cells. By clonal selection of viable cells which have accumulated

genetic damages, normal mucosa cells ultimately evolve into malignant mucosa cells over an indefinite period (Califano *et al.* 1996b). The developing of malignant oral mucosa cells is life-threatening and always lead to a poor prognosis.

The prognosis of oral cancer is highly affected by the location, stage, and grade of neoplastic tissue. The tumor stage is an important prognosis predictor. According to the American Joint Committee on Cancer (AJCC), the 5-year survival rate for oral cancer is as follows in descending order of survival rate: stage I, 65 to 70%; stage II, 50 to 55%; stage III, 38 to 44%; and stage IV, 25 to 29% (Greene *et al.* 2002). The histological grade of oral cancer does have independent prognostic value. Grade I, II, and III of tumor mass are termed as well, moderately, and poorly-differentiated. Poorly-differentiated tumors have a poorer prognosis overall than well-differentiated, with a higher propensity of lymph node metastasis. Five-year survival rates of well-differentiated, moderately differentiated, and poorly differentiated tumors are reported as follows: grade I, 54 to 80%; grade II, 41 to 62%; grade III, 29 to 70% (Kademani *et al.* 2005).

Besides tumor staging and grade, biomarkers are also emerging as valuable prognostic markers. Numerous studies have shown the prognostic significance of different biomarkers in tissues, which include p53, p27, Ki-67, and proliferating cell nuclear antigen (Bettendorf *et al.* 2004). Low survival rate of stage III and stage IV oral cancer is mostly due to regional lymph node metastasis and high recurrence incidence leading to ineffectiveness of current treatment modalities and regimes. As a result, a conceivable strategy to eradicate oral cancer is early detection of oral cancer followed by effective treatments at its early stage.

## 2.4 Oral Pre-cancer and Malignant Transformation

Fortunately, benign oral precancer lesions are visible in the oral cavity. Its good visibility offers a chance for early detection of oral cancer. Oral cancer is commonly preceded by oral precancer which is defined as ‘a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart’ (Kramer *et al.* 1978). Well-recognized oral precancer lesions and conditions that are associated with malignant potential include leukoplakia, erythroplakia, lichen planus and submucous fibrosis.

Oral leukoplakia is defined as a white plaque that cannot be scraped off in the oral cavity (Kramer *et al.* 1978). Oral erythroplakia normally appears as a well-demarcated red plaque with a soft, bright-red, velvety texture. Oral lichen planus is an autoimmune, T-cell mediated condition that gives rise to the accumulation of lymphocyte beneath basal keratinocytes and hyperkeratosis (Lodi *et al.* 2005). Oral submucous fibrosis is an irreversible, chronic disease with the earliest symptom being burning sensation while eating spicy foods followed by blanching and hardening of the mucosa resulting in restriction of mouth opening (Pundir *et al.* 2010). Oral leukoplakia is more common than erythroplakia while erythroplakia is prone to develop into invasive carcinoma (Reichart and Philipsen 2005). All types of precancerous lesions aforementioned are intermediate steps towards transformation into malignant lesions.

Generally, oral precancer malignant transformation rate varied from 14% to 50% with weighted average 26.3% in the studies follow-up less than 2 years and more than 10 years (Reichart and Philipsen 2005). Oral erythroplakia has the highest potential of malignant transformation compared to other precancerous lesions and conditions (Reichart and Philipsen 2005). A review of recent studies showed that the malignant

transformation rate of oral leukoplakia was varied from 8.9 to 17.5% (Neville and Day 2002). A 7.6% malignant transformation rate of oral submucous fibrosis was observed over a 17-year period (Murthi *et al.* 1985). The transformation rate of oral Lichen Planus was estimated to be 1% over 5 years (Lodi *et al.* 2005). Understanding of transformation rate of clinically distinct lesions may facilitate clinicians to better weigh the benefit and risk of treatments of cancer patients.

## **2.5 Diagnosis and Early Detection of Pre-malignant Lesions**

Early detection of any pre-cancerous and early malignant oral lesions by visual examination is more clinically feasible and practical. This is because the easy accessibility of the oral cavity and the apparent clinical presentation of premalignant lesions. However, a tissue biopsy of precancerous lesions with a gold standard histopathologic review is strictly required for the evaluation of oral dysplasia or invasive carcinoma in order to make a definitive diagnosis.

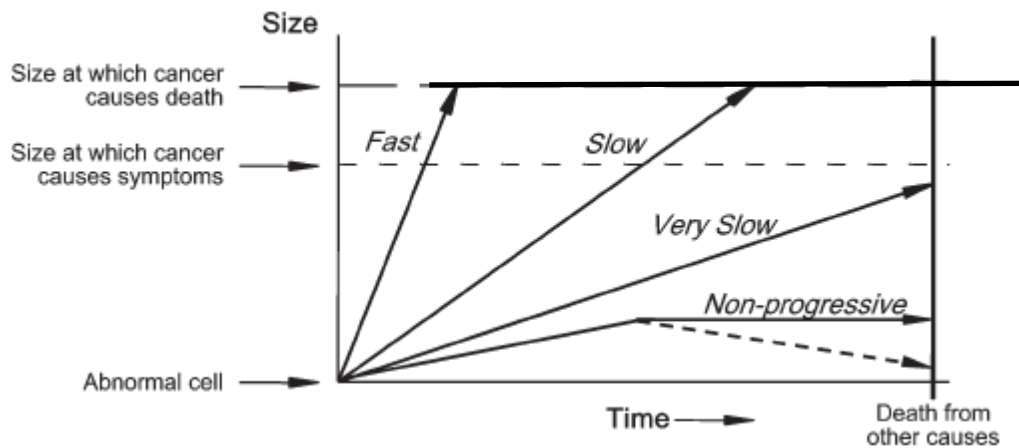
Several adjuvant diagnostic aids have been used to assist in visual recognition and detection of oral mucosa precancerous lesion sites in the oral cavity. These aids include toluidine blue staining, oral brush biopsy (OralCDx), ViziLite, and oral autofluorescent. Toluidine blue stains the DNA and RNA and therefore dysplastic and malignant cells that contain higher quantities of DNA and RNA are preferentially stained dark blue. OralCDx enables dentists to obtain exfoliated dysplastic tissues that are covered underneath a thick layer of keratin from a suspected site. With the ViziLite kit, chemiluminescent light is used to inspect the oral cavity of patients in a dimly lit room after mouth washing with acetic acid. Abnormal lesions which are highly keratinized will appear white while normal mucosa should appear dark. Oral

autofluorescent is a novel technology that examines the autofluorescent signals emitted from biomolecules in cells particularly NADH and FAD molecules. Dysplastic epithelial cells which lack of NADH and FAD activities will emit less autofluorescent signal compared to normal cells resulting in abnormal lesions appearing dark (Patton *et al.* 2008; Jerry *et al.* 2010).

With the mentioned early-detection technologies, a declining of incidence and mortality rate of oral cancer is still not significantly observed in Malaysia. In addition, most patients with oral cancer are diagnosed when they are already at the advanced stage where prognosis is generally bad (Ariffin and Saleha 2011). This is perhaps due to the delay in diagnosis of oral cancer. The reasons for the delays in diagnosis have been studied by Khoo SP. at five specialist centres in Malaysia from 1994 – 1995. The results showed that asymptomatic premalignant lesions and delays in obtaining biopsy reports are the main reasons for late diagnosis (Khoo *et al.* 1996). Therefore, it is essential to educate people with regards to the characteristics of oral premalignant lesions and plausible risk factors of oral cancer (Neville and Day 2002). Furthermore, there is a pressing need to identify reliable molecular factors which are able to diagnose oral cancer early and requires a shorter time for analysis. These biomarkers has been suggested as the risk factors for determining the malignant transformation rate of oral premalignant lesions in order to aid clinicians planning the management strategies and therapies for their oral precancer patients (Lozada-Nur 2009).

However, early detection of cancer may unintentionally lead to overdiagnosis which is a diagnosed condition that would not go on to cause death or symptoms (depicted in Figure 2.2). Raising diagnostic threshold of cancer screening has been proposed to mitigate overdiagnosis (Welch and Black 2010). It is believed that a new

insight in cancer associated molecular markers will ultimately allow us to accurately predict tumor behavior and thus address overdiagnosis.



**Figure 2.2:** Tumor size increasing along cancer progression timeline. Early detection of cancer below tumor size at which cancer causes symptoms may lead to overdiagnosis (dotted line). This is because those cancers may grow very slowly till their host death from other causes or they never grow to cause cancer symptoms and even regress afterward. **Source of reference:** Welch, H. G., & Black, W. C. (2010). Overdiagnosis in cancer. *J Natl Cancer Inst* 102(9): 605-13.

## 2.6 Early Cancer Molecular Markers

Biomarkers can be derived from DNA, RNA, or proteins. Nucleic acid based markers which are freely circulating in bodily fluids are worthwhile for screening. Cancer associated gene mutations are primary targets as early cancer markers such as TP53 mutation. Mutation of TP53 has been used in tracking the status of head and neck cancer lymph node metastasis (Sidransky 2002). Moreover, loss of heterozygosity or microsatellite instability was examined in head and neck squamous cell carcinoma

(HNSCC) primary tumors and saliva samples and at least one of the alteration markers has been detected in 86% of primary tumors and 79% of saliva samples (Nawroz *et al.* 1996). DNA methylation which is involved in silencing tumor suppressor genes has also been detected in saliva samples of oral cancer (Rosas *et al.* 2001). HPV infection has been shown to be associated with oral cancer initiation as well. Hence, detection of HPV DNA provides an alternative strategy for early marking of risky groups (Sidransky 2002).

Although carcinogenesis is always due to genomic mutations, examination of the proteome gives more crucial information than analysis of nucleic acids or the genome independently (Banks and Selby 2003). Therefore, protein biomarkers may be more useful in predicting the invasiveness and occurrence of cancer. In theory, differential expression of proteins can be predicted from the analysis of the transcriptome or translome in response to internal and external events due to the fact that proteins are translated from mRNA. However, there is a discrepancy when comparing the results of transcriptomic and proteomic analyses of human cancer.

Due to the fact that proteins are the biologically functional end-units of the genome, a study of the proteome should provide more meaningful insights into cell interaction and signaling in cancer. Moreover, proteins undergo posttranslational modifications and proteolytic cleavage upon translation and transcription from RNA and DNA. These modifications are not reflected in the genome and transcriptome. Thus, proteomic analysis is more useful in identifying biomarkers (Mlynarek *et al.* 2007).

By proteomic approaches, a number of potential protein biomarkers for oral cancer have been identified and they include telomerase, Mac-2 binding protein and MAGED4B which were over-expressed in OSCC tissues (Califano *et al.* 1996a; Weng



*et al.* 2008; Chong *et al.* 2012). These protein markers may have specific roles in carcinogenesis to promote cancer cell growth and survival. Therefore, when a cell is aberrantly expressing these protein biomarkers, it would be an omen of cancer transformation and initiation.

## 2.7 Cancer Molecular Biology and Tumor Microenvironment

Irreversible genetic mutations induced by carcinogens or viruses in a single cell are a cornerstone of cancer initiation. Subsequent cancer proliferation with accumulated genetic mutations and more epigenetic changes promote cancer progression and further hyperplasia. Ultimately, localized cancer cells evolve into malignant cancer endangering the host's life. Cancer development and progression are regulated stepwise through activating oncogenes and silencing of tumor suppressor genes. Oncogenic proteins such as platelet derived growth factor (PDGF), epidermal growth factor receptor (EGFR), Ras proteins, c-myc transcription factor, and bcl-2 antiapoptotic proteins promote cancer cells to proliferate indefinitely while repress cell senescence and apoptosis. Moreover, deregulation of tumor suppressor genes such as hMSH2, RB proteins, and p53 resulting in more genetic instability, uncontrolled cell proliferation, and evasion of programmed cell death (Wong *et al.* 1996; Bertram 2001).

Cancer cell hyperproliferation and immortalization are fundamental in cancer transformation. Loss of cell cycle control is the key step leading to hyperproliferation independent of growth signals. Deregulation of cell cycle associated proteins such as KIP family (p21, p27, p57), INK4 family (p16, p15, p18, p19), cyclin D1, Ras proteins and RB proteins causes cancer cells undergo unlimited mitosis via unguarded restriction point (R) of the cell cycle and transit from G1 to S phase rather than rest in G0 phase.

Proliferation of cancer cells is also enhanced by over-expression of mitogenic factors such as PDGF and epidermal growth factor (EGF) (Roussel 1999; Bertram 2001; Greene *et al.* 2002).

As the size of tumor increase due to cancer cells proliferation, size expansion may cause the tumor to suffer from a hypoxic condition which can limit the cancer cell growth. This condition is especially prominent at the centre of the tumor mass. However, oxygen supply can be made available by formation of new blood vessels inside the tumor mass. Tumor secretion of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) is vital for the stimulation sprouting of new blood vessel formation from nearby blood vessels towards the centre of tumor mass (Carmeliet and Jain 2000).

When the normal cells replication numbers reach a critical limit, they are preordained for cell apoptosis or are cell cycle arrested. Such phenotype was first coined by Hayflick and thus termed as the “Hayflick limit” (Hayflick 1965). The loss of replicative potential of normal cells has been attributed to telomere shortening with each subsequent cell doubling. The telomere is a repeated 5'-TTAGGG-3' hexanucleotide sequence with a t-loop end at the terminal of chromosomes. This telomeric sequence confers protective effects on chromosome ends from being recognized as damaged DNA or DNA double-strand breaks which may lead to programmed cell death and senescence. In order to preserve the replicative potential of cancer cells, immortalized cancer cells have developed a special cellular mechanism to maintain their telomere length and chromosome stability by expressing telomerase reverse transcriptase or via homologous recombination between chromosome ends (Chiu and Harley 1997).

Maintaining telomere length has been proved important in breast and colon cancer cell growth (Hahn *et al.* 1999).

Programmed cell death or apoptosis is a fail-safe mechanism to eradicate normal cells which have irreparable DNA damage from surviving and transforming into cancer cells. One particular tumor suppressor gene, p53 has been intensely studied in its role in the activation of apoptosis by inducing mitochondrial release of cytochrome c into the cytosolic space. Unleashed cytochrome c molecules cluster to Apaf1-procaspase 9 complexes resulting in activation of diverse caspase protease family members. These caspase proteases degrade all intracellular proteins or activate other apoptotic-associated proteins (Amaral *et al.* 2010). Moreover, a p53-independent apoptosis pathway can be initiated via death receptors known as FasR/CD95R , TNF receptor and Trail receptor. When their respective ligands bind to receptors, a downstream signaling pathway activates caspase 8 which in turn perforate the mitochondria to release cytochrome c into the cytosol. Therefore, most cancer cells lose their wild-type p53 function via gene mutation, downregulation of death receptors, and overexpression of decoy death receptors to evade apoptosis (Ozoren and El-Deiry 2003). In addition, overexpressed apoptosis inhibitors such as Bcl-2 and Mdm-2 show synergistic effect on cancer cells to improve their survival and become more apoptosis resistant (Iwakuma and Lozano 2003; Amaral *et al.* 2010).

Cancer cells dissemination is a fatal progression. It involves cellular morphology alterations and motility enhancements. Initially, cancer cells undergo epithelial to mesenchymal transition (EMT) to be more invasive and mobile (Huber *et al.* 2005). This is followed by intravasation where the cancer cells penetrate through the basement membrane of blood vessels and diffuse into the blood stream for transportation to a

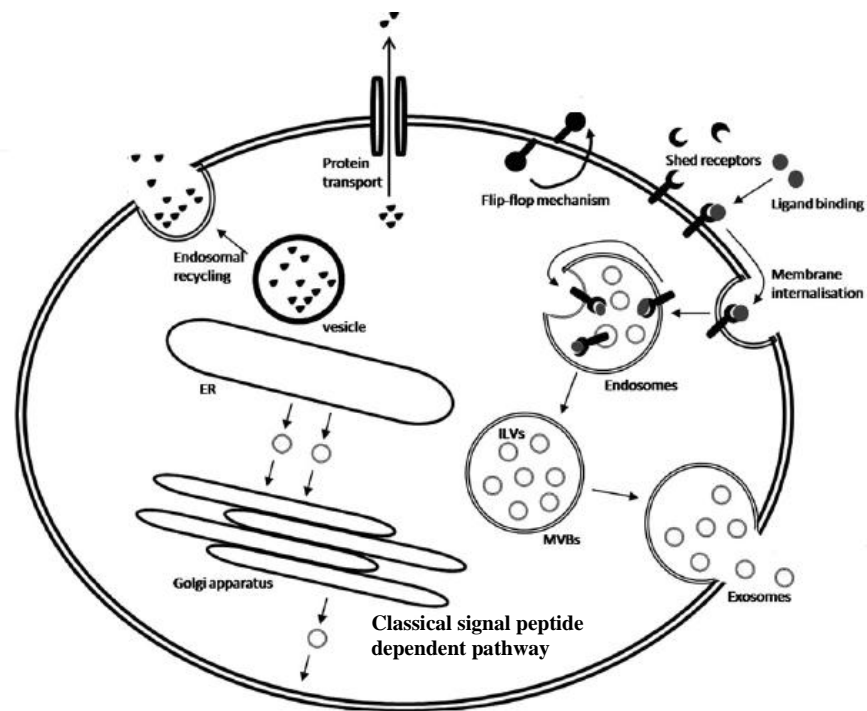
distinct site. When the cancer cells are arrested in the capillaries of a new site, the cancer cells begin to migrate across the slit of endothelial membrane of blood vessel into the remote secondary site. Nevertheless, several barriers will be encountered during cancer dissemination. Such obstacles are immune surveillance in blood stream and blockade of extracellular adhesion molecules leading to inefficiency in cancer metastasis. Hence, the tumor microenvironment plays a vital part in facilitating cancer metastasis and improving its metastasis efficiency (Chambers *et al.* 2002).

In order to evade T-cell mediated immunity, major histocompatibility complex (MHC) membrane proteins of cancer cells are down-regulated. By contrast, expression of MHC surrogates in cancer cells are up-regulated to inhibit natural killer cell cytotoxicity which is elicited by “missing self” cells phenotype (Igney and Krammer 2002). Cancer cells and its neighboring cells like fibroblast and stromal cells secrete a diverse variety of protease families like matrix metalloproteases (MMPs), a disintegrin and metalloproteases (ADAMs), serine proteases, cathepsin cystein proteases into the tumor microenvironment. These proteases degrade adhesion molecules and extracellular matrix components including laminin, collagen, and fibrin that impede the movement of metastatic cancer cells. Additionally, cleaved fragments of extracellular matrix will become stimulators of angiogenesis. Degradation of ECM may also lead to liberation of trapped growth factors such as FGF and VEGF into the tumor microenvironment for sustaining growth and facilitating angiogenesis (Chambers *et al.* 2002; Bogenrieder and Herlyn 2003; Friedl and Alexander 2011). Therefore, studying the proteome of tumor microenvironment may provide insights on their roles in cancer metastasis as well as discovers any potential diagnostic and therapeutic protein targets.

## 2.8 Secretome-based Proteomics

Secretome of cancer cells is an integral part of the tumor microenvironment. The term "secretome" was first proposed by Tjalsma et al. in a genome-based survey on secreted proteins of *Bacillus subtilis* (Gronborg *et al.* 2006). The secretome refers to secreted peptides or proteins which include enzymes and the extracellular matrix protein components. They are expressed from only about one-tenth of the human genome. These extracellular peptides or proteins can be trafficked out via exosome, endosomal recycling, actively protein transport, flip-flop mechanism, or classical signal-peptide dependent pathway (depicted in Figure 2.3). Shedding of the ectodomain of transmembrane protein from cancer cells introduces more complexity into the cancer secretome protein profile. This is interesting that some cancer associated intracellular proteins are transported out into the cancer secretome while their exact function in carcinogenesis remain elusive (Karagiannis *et al.* 2010).

The cancer secreted proteins are likely growth factors, ECM remodeling molecules, cytokines or other bioactive molecules which are vital in the differentiation, proliferation, metastasis and angiogenesis of cancers. For example, they may include probably osteopontin, galectin-3, transforming growth factor- $\beta$  (TGF- $\beta$ ), and matrix metalloproteinase (Xue *et al.* 2008; Mbeunkui and Johann 2009). As this group of proteins has an essential relationship with carcinogenesis, there will be a high likelihood of uncovering novel biomarkers in the cancer secretome.



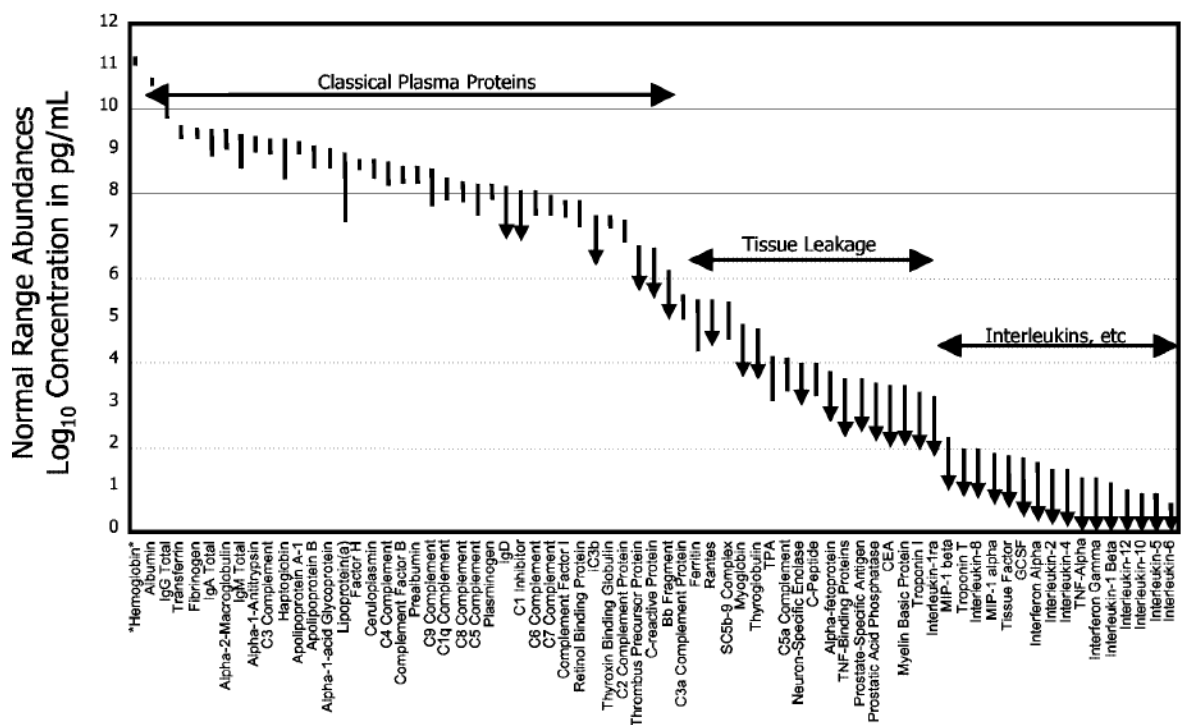
**Figure 2.3:** Protein secretory pathways. **Source of reference:** Karagiannis, G. S., Pavlou, M. P., & Diamandis, E. P. (2010). Cancer secretomics reveal pathophysiological pathways in cancer molecular oncology. *Mol Oncol* 4(6): 496-510.

## 2.9 Challenges in Resolving the Cancer Secretome from Plasma

Secreted protein biomarkers for cancer proteomic analysis can be obtained from a number of sources such as cancer cell lines, human tissues, and body fluids such as plasma (Chen and Yates 2007). Plasma proteomics is an attractive approach to discover any potential secreted biomarkers which reflects the status of the body instantly. In addition, it is also the most accessible sample from patients (Omenn *et al.* 2005). However, plasma is very complex and this creates problems when attempting to perform a proteomics based analysis. The large dynamic concentration range of up to 12 orders of magnitude for plasma proteins, as depicted in Figure 2.4 masks the lower

abundance proteins. It is particularly so for secreted proteins which are low abundance and are present in below microgram per milliliter concentration. This introduces a major challenge in performing a comprehensive plasma proteome analysis (Anderson and Anderson 2002). The most widely used proteomics approach, 2D gel electrophoresis (2DE) has a range of detection of not more than 3 orders of magnitude. Thus, it is unable to resolve low abundance proteins which are located below the lower limit of its detectable range (Omenn *et al.* 2005).

As a result, removal of high abundance proteins is an important step to reveal the low abundance proteins in the sample. High abundance proteins in plasma can be removed through enrichment steps such as the use affinity columns. However, this lowers the screening efficiency due to possible nonspecific binding of proteins to the column itself (Yocum *et al.* 2005). Furthermore, albumin (the most abundant protein in the plasma) is a carrier protein and thus removal of this protein may cause loss of valuable information in the form of proteins that may be removed along with the removal of albumin.



**Figure 2.4:** Plasma protein abundance range with reference interval for 70 protein analytes. This graph is plotted on a log scale spanning 12 order of magnitude. **Source of reference:** Anderson, N. L., & Anderson, N. G. (2002). The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 1(11): 845-67.

## 2.10 Strategy for Bypassing the Problem of Plasma Proteins Dynamic Range in Proteomics

Due to the limitations of plasma proteomics that has been described here, a comparison of secreted protein patterns in human cancer tissue with their normal counterparts can be employed to overcome these limitations. However, the amount of human tissues and inherent variations among individual patients can be limiting factors for clinical proteomic studies. As a result, a study of the secretome using human tissue must be carefully manipulated to differentiate true clinical differences in protein



expression from variation in sample collection, experimental condition, and normal biological variability (Chen and Yates 2007). This makes such a study complex and time consuming.

Alternatively, cancer cell lines can be used as a surrogate in the study of the cancer secretome. Cancer cell lines originated from different anatomic site have distinct molecular expression pattern leading to poor therapy outcome (Severino *et al.* 2008). In order to minimize molecular heterogeneity of cell lines from different anatomic sites, cancer cell lines from the same anatomic site were selected as studying subjects in this project. As cancer cell lines are cultured under controlled conditions, they exhibit a high degree of homogeneity of samples and provide a large amount of sample. However, cancer cell lines may undergo genotypic and phenotypic drifts during the process of cell culture due to clonal selection. Therefore, it will be necessary to perform validation for *in vivo* physiological relevance. In the validation process, immunoassays which are based on specific antibody and antigen reaction are commonly employed. This is due to its higher sensitivity and specificity to detect low abundance promising secreted biomarkers which may be present in the bloodstream and interstitial fluid of cancer tissues (Xue *et al.* 2008). While, real-time quantitative PCR can be carried out for preliminary biomarkers verification due to its lower cost and high sensitivity.

The major barriers to this surrogate strategy are difficulties in establishing the primary culture of normal epidermal keratinocytes from the resected normal mucosa tissues. This is in part due to the unsustainable growth of normal primary culture and their limited lifespan. Additionally, it is necessary to limit the degree of cell lysis as the cancer cell lines are an *in vitro* model and contamination of cytosolic proteins during cell lysis will contribute towards an inaccurate view of the secretome. Nevertheless,

cytosolic and nuclear proteins may also naturally be transported into extracellular space from microvesicular bodies (known as exosome), cell leakages and apoptotic blebs which exhibit pleiotropic biological functions during cell growth (Mathivanan *et al.* 2010). As a result, some cytosolic and nuclear proteins may combine with classical secreted proteins giving rise to a distinct version of the secretome. Despite these limitations, secretome preliminary studies using cancer cell lines is relatively lower cost, easy-to-use, and is an unlimited sample sources compared to using human tissues (Chen and Yates 2007).

## 2.11 Gel-Based Proteomics

Gel-based proteomics coupled with mass spectrometry is usually employed in the analysis of complex mixtures of secreted proteins from cancer cell lines. Two dimensional gel electrophoresis which was developed in mid 1970's (O'Farrell 1975) is used in resolving hundreds to thousands of heterogeneous proteins in a single polyacrylamide gel. It is based on the isoelectric point of proteins to separate them by using immobilized pH gel gradients. This is followed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS PAGE) to further separate the proteins on the basis of their molecular weight. As a result, a map of protein spots can be visualized in a single gel where each spot represents a different protein, different isoforms of the same protein, or its post-translational modifications upon staining (Alessandro *et al.* 2005). Subsequently, identification of differentially expressed proteins is performed by tandem mass spectrometry. With this technology, tryptic peptides of digested protein spot are sorted based on their mass to charge ratio in a mass spectrum. Each detected tryptic peptide mass will be subsequently fragmented by collision-induced dissociation (CID)

individually. This will generate a fragmented ions mass spectrum which is representation for a unique type of protein. The protein identity can be deduced by matching this spectrum with theoretical spectrum in a well-established database (Xue *et al.* 2008).

However, it has several pitfalls such as potential difficulties in the identification of proteins with extreme isoelectric points or molecular weight, and low sensitivity in the detection of low abundance proteins and hydrophobic membrane proteins. In addition, this technique is time-consuming and labor-intensive (Monteoliva and Albar 2004). A modified 2DE method, differential in-gel electrophoresis (DIGE) reduces experimental variations but it still has several shortcomings such as the fact that it is inapplicable for proteins without lysine residues (Xue *et al.* 2008). In spite of these inherent pitfalls, two-dimensional gel electrophoresis coupled to mass spectrometry is still a robust and reliable approach in preliminary discovery of potential biomarkers.

**CHAPTER 3**

**MATERIALS**

**&**

**METHODS**

### 3.1 Normal Tissues Collection and Establishment of Normal Primary Culture

#### 3.1.1 Materials:

Extraction media (10 mL DMEM-F12 medium, 10% FBS, 200 iu/mL penicillin, 200 µg/mL streptomycin and fungizone, 5 µg/mL)

Absolute ethanol

Phosphate Buffer Saline

Keratinocyte serum free media (KSFM, Invitrogen) containing 200 iu/ml penicillin, 200 µg/ml streptomycin, 0.4 ng/ml epithelial growth factors (EGF), 25 µg/ml bovine pituitary extract (BPE) and 30 µM calcium chloride

Trypsin type III (Sigma)

#### 3.1.2 Methods:

Normal tissues were derived from surgically resected tissue specimens from different impacted tooth patients at the Dental Faculty and University Malaya Medical Centre, Kuala Lumpur Hospital. Informed consent was obtained from all normal tissue donors prior to surgery. This project was approved by the Medical Ethics Committee, Faculty of Dentistry, University Malaya and endorsed by the Ministry of Health, Malaysia (Medical Ethics Clearance no. DF OP1006/0041(P)). Information regarding the donors and from which the primary cultures were derived are tabulated in Table 3.1. Tissues were collected in 30-mL extraction media immediately upon excision of the normal tissues.

The tissues were soaked in absolute ethanol for 20~30 seconds followed by washing twice with PBS under sterile conditions. It was incubated in 2.5 mL of 1%

trypsin type III PBS containing 200 iu/mL penicillin and 200 µg/mL streptomycin at 4 °C for 16 hour and then incubated at 37 °C for 30 minutes. Five milliliters of DMEM-F12 with 10% FBS was added and the mixture was pipetted vigorously to disaggregate the cells in the tissues. The cells were then pelleted at 3000 rpm and washed in PBS twice at 1000 rpm for 10 minutes. Subsequently, the cell pellets were re-suspended in KSFM and seeded into 25 cm<sup>2</sup> culture flasks (Hamid *et al.* 2007). Epidermal keratinocytes were selectively grown from 4 individual tissues using protocols above. Subsequently they were cultured into 4 primary cultures (316N, 317N, 322N, 326N) with maximum 4 to 6 passage numbers only for the secretome studies. In addition, they are derived from the same anatomic site (mouth gum) to reduce molecular heterogeneity among the samples in comparative proteomic studies.

**Table 3.1:** Details of donors for normal primary cultures

<b>Normal Primary Culture</b>	<b>Age</b>	<b>Gender</b>	<b>Race</b>	<b>Anatomic site</b>	<b>Risk habits</b>
316N	34	Female	Malay	Gum	None
317N	26	Female	Chinese	Gum	None
322N	27	Female	Malay	Gum	None
326N	26	Male	Malay	Gum	Smoking

## **3.2 MTT Viability Test on Cancer Cell Lines for Treatment of Serum-free Media Over 24 and 48 Hours Incubation**

### **3.2.1 Materials:**

DMEM-F12 medium (Invitrogen)

Fetal bovine serum (FBS)

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Incubator for cell cultures

Plate reader

Dimethyl sulfoxide (DMSO)

### **3.2.2 Methods:**

Cancer cell lines were preferred to be studied due to their cell clonal homogeneity. Two well-established oral cancer cell lines, 48T and 153T were selected from the oral cancer cell lines depository of Cancer Research Initiative Foundation (CARIF) as study subjects. Another selected oral cancer cell line, H400 was a kind gift from Professor Stephen Prime, University of Bristol. The details for cancer cell lines are tabulated in Table 3.2. Cultured cell passage numbers of 48T, 153T, and H400 used for this study were 26, 25, and 28 respectively. With such moderately low passage numbers of cell lines, genetical or phenotypical drift of cell lines perhaps would be very little and negligible.

Approximately 5000 cells were seeded in each well of a 96-well plate. All wells with seeded cells were incubated in DMEM-F12 medium with 10% fetal bovine serum

for 18 hours. The media of each well was then replaced with serum free media and incubated for 24 hours and 48 hours respectively. Following the incubation period, serum free media was discarded and 20  $\mu\text{L}$  of MTT was added into the well for 4 hours in a 5%  $\text{CO}_2$  incubator. MTT was then removed and 100  $\mu\text{L}$  DMSO subsequently added. Absorbance of each well was read using a plate reader at 575 nm.



**Table 3.2:** Details of cancer cell lines been studied

Cancer cell lines (passage numbers)	Age	Gender	Race	Anatomic site	Clinical TNM (stage)	Pathologic TNM (stage)	Risk habits
48T (26)	77	Female	Sikh	Gum	-	T4N2aMx <sup>a)</sup> (IV)	None
H400 (25)	55	Female	Caucasian	Gum	T2N0M0 <sup>b)</sup> (II)	-	* <i>Paan</i> Chewer; smoking
153T (28)	36	Male	Indian	Gum	T4N2aM0 <sup>c)</sup> (IV)	T4N2bM0 <sup>d)</sup> (IV)	Smoking

**Footnotes of Table 3.2:**

- a) Tumor invades adjacent structures; Metastasis in single ipsilateral lymph node; Presence of distant metastasis cannot be assessed
- b) Tumor size more than 2 cm and less than 4 cm; No regional lymph node metastasis; No distant metastasis
- c) Tumor invades adjacent structures; Metastasis in single ipsilateral lymph node; No distant metastasis
- d) Tumor invades adjacent structures; Metastasis in multiple ipsilateral lymph nodes; No distant metastasis

\* A kind of preparation of betel leaf combined with areca nut or tobacco

### 3.3 Preparation of Secretome Sample

#### 3.3.1 Materials:

DMEM-F12 media (Invitrogen)

Keratinocyte serum free media (Invitrogen)

Casy<sup>®</sup> Model DT cell counter (Innovatis)

Amicon Ultra-15 (Millipore)

#### 3.3.2 Methods:

Cancer cell lines and normal primary cultures were grown in DMEM-F12 media (with 10% FBS) and KSFM respectively until 75% confluency. Old culture media were drawn out from culture flask and washed extensively with PBS for 5 times. Both cancer cell lines and normal cell lines were then cultured in serum-free DMEM-F12 media for 24 hours. After 24 hours, approximately 10 mL of conditioned medium was harvested and centrifuged at  $1000 \times g$  to remove cell debris and floating dead cells. Subsequently, living cells were harvested into a microcentrifuge tube by trypsinization and centrifugation at  $1000 \times g$ . Total cell number and cell death were measured by Casy<sup>®</sup> Model DT cell counter. Conditioned medium was then concentrated and desalted using Amicon Ultra-15 ultracentrifuge tube (cut off 3 kDa).

### 3.4 Protein Quantitation - Bradford assay

#### 3.4.1 Materials:

Bovine serum albumin (BSA) standard solutions (prepared by using serial dilution):

Stock solution **A** (1.00 mg/mL),

Standard solution **B** (10.00 µg/mL),

Standard solution **C** (5.00 µg/mL),

Standard solution **D** (2.50 µg/mL),

Standard solution **E** (1.25 µg/mL),

Standard solution **F** (0.625 µg/mL)

Bradford reagent from Biorad

Ultrospec™ 2100 pro UV/Visible Spectrophotometer from Amersham Bioscience

#### 3.4.2 Methods:

A total volume of 800 µl of each standard solutions **B**, **C**, **D**, **E**, and **F** was pipetted into a dried and clean test tube. Subsequently, Bradford dye reagent, 200 µl was added into each test tube. It was then mixed thoroughly by vortexing. The mixture was incubated at room temperature for 5 minutes. Absorbance was read at 595nm for each of the standard solutions. A standard curve (absorbance against protein concentration) was constructed. The same procedure was performed for samples.

### **3.5 The First Dimension: Isoelectric Focusing of Proteins (pH 3 – 10 Linear)**

#### **3.5.1 Materials:**

Dithiothreitol (DTT)

24 cm Immobiline Drystrip from GE healthcare

Immobiline Drystrip Cover Fluid

Rehydration solution ( 8M urea, 2% w/v CHAPS, 0.5% v/v Pharmalyte or IPG buffer pH 3-10, 0.002% bromophenol blue )

2-D Clean-up kit from Amersham Bio-rad

Ettan IPGphor 3 from GE Healthcare

#### **3.5.2 Methods (rehydration of IPG strips):**

Samples were thawed and treated with 2-D Clean-up kit to remove any impurities and salts in the sample according to the manufacturer's instruction. Subsequently, 28 µg of DTT was added into 1 ml of thawed rehydration solution. Then, 50 µg of sample (protein) were mixed with DTT added rehydration solution to give a final volume of 450 µl. 450 µl of rehydration which contained DTT and sample proteins was loaded onto slot in the reswelling tray. Any large bubbles were removed. The code of the strip gel was recorded and assigned to a specific loaded sample. An Immobiline DryStrip was placed in the reswelling tray channel with gel side down. The gel strip was gently pressed to remove any trapped bubbles. The strip was then overlaid with Immobiline DryStrip Cover Fluid and rehydrated for 18 hours.

### 3.5.3 Methods (Isoelectric Focusing of Proteins):

Two electropads were cut to an appropriate width and length. IPG strips were taken out of the reswelling tray and excess water was removed with filter paper. The strips were then placed into the strip holder and wet electropads were placed on both ends of the gel strip. Immobiline DryStrip cover fluid was then overlaid on the strips. The Isoelectric focusing unit, Ettan IPGphor 3 was programmed with the following parameters:

Stage	Voltage mode	Voltage (V)	Time (h:min)	kVh
1	Step and hold	500	1:00	0.5
2	Gradient	1000	1:00	0.8
3	Gradient	8000	3:30	13.2
4	Step and hold	8000	6:52	55.0
Total			12:22	70.0

Upon completion of the run, IPG strips were removed from the strip holder and rinsed gently with ddH<sub>2</sub>O. The IPG strips were then stored in screw-cap tubes at -20 °C up to 1 month. At this point, the strips were ready for second-dimension electrophoresis.

### 3.6 Preparation of Homogenous Vertical SDS Slab Gels

#### 3.6.1 Materials:

4x Resolving gel buffer (1.5M Tris-HCl at pH 8.8)

Ammonium persulfate solution (10% w/v)

Tetramethylethylenediamine, TEMED

SDS (10% w/v) stock solution

30% T, 2.6% C monomer stock solution (30% w/v acrylamide, 0.8% w/v N,N'-methylene bis-acrylamide)

Overlay buffer (0.375M Tris-HCl at pH 8.8, 0.1% w/v SDS)

Gel-casting apparatus from GE Healthcare, USA

#### 3.6.2 Methods:

Glass plates were rinsed with ddH<sub>2</sub>O and dried with Kimwipes. They were then cleaned with Kimwipes soaked in ethanol and allowed to air dry. The gel-casting unit was assembled described by the manufacturer (General Electronic Healthcare). A 13%T of resolving gel mixture for two slab gels was prepared as follows:

<b>Reagents</b>	<b>Volume</b>
Acrylmide	34.7 mL
4x resolving gel buffer	20.0 mL
ddH <sub>2</sub> O	24.1 mL
10% SDS	800.0 $\mu$ L
10% APS	444.4 $\mu$ L
TEMED	21.3 $\mu$ L
<b>Total</b>	<b>80 mL</b>

The solution was mixed well prior to the addition of TEMED. The gel solution was then slowly poured slowly into the assembled gel tank up to a level ~1.0 – 1.5cm below the top of the glass plate. The gel was overlaid with a thin layer of 0.1% SDS solution. The gels were allowed to polymerize. Polymerized gels were stored at 4 °C until use.

### 3.7 IPG Strip Equilibration

#### 3.7.1 Materials:

SDS equilibration buffer (6M urea, 75mM Tris-HCl pH8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, total 200ml)

Shaker table

Dithiothreitol (DTT) from Merck, USA

Iodoacetamide (IAA) from Merck, USA

#### 3.7.2 Methods:

First, 100 mg of DTT was dissolved in 10 ml of SDS equilibration buffer resulting in final DTT concentration of 1% w/v. Then, ten milliliter of DTT – SDS equilibration buffer solution was delivered into the IPG strip holding tube. The IPG strip holding tube was gently shaken for 15 minutes. After 15 minutes of elapsed time, 0.25 g of iodoacetamide was dissolved in 10 mL of SDS equilibration buffer for a second equilibration step. Upon completion of the first equilibration step, the equilibration solution was decanted and the second equilibration buffer was delivered into the IPG strip holding tube. The IPG strip was again gently shaken for 15 minutes. After completion of the second equilibration step, excess equilibration buffer was drained with a filter paper. The IPG strip was now ready for second dimensional gel electrophoresis.



### **3.8 The Second Dimension: SDS – Gel Electrophoresis of Proteins**

#### **3.8.1 Materials:**

Agarose sealing solution (25mM Tris Base, 192mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue, total 100ml).

10x Running buffer (250mM Tris base, 1.92M glycine, 1% SDS)

Ettan Dalt II separation unit from Amersham Bioscience

Forceps

Focused IPG strip

#### **3.8.2 Methods:**

A sufficient amount of agarose sealing stock gel was scooped into a beaker. Subsequently, it was melted in microwave oven. Once melted, it was allowed to cool down to approximately 45 °C. A pair of forceps was used to position the equilibrated IPG strip on the surface of the second-dimension gel. The warmed agarose solution was poured onto the vertical second-dimension gel gently. The melted agarose solution was allowed to cool and solidify. Second-dimension electrophoresis was performed firstly at constant 2 W for 45 minutes. The wattage was then raised to 110 W. The power supply was turned off when the bromophenol blue dye front reached the bottom of the gel. Gel fixation was immediately performed upon completion of the run.

### 3.9 Silver Staining

#### 3.9.1 Materials:

Fixing solution ( 40% v/v ethanol, 10% v/v acetic acid )

Sensitizing solution ( 12.65 mM sodium thiosulfate, 25% w/v glutaraldehyde, 0.83 M sodium acetate, 0.3% v/v ethanol )

Silver solution ( 14.72 mM silver nitrate, 0.015% v/v formaldehyde )

Developing solution ( 0.24 M sodium carbonate, 0.015% v/v formaldehyde )

Stopping solution ( 0.05 M EDTA acid )

Preserving solution ( 30% v/v ethanol, 4% v/v glycerol)

Stainless steel tray

Orbital shaking platform

#### 3.9.2 Methods:

Upon completion of the second dimension SDS-PAGE, gels were transferred into a stainless steel tray which was filled with fixing solution. Fixing was performed overnight.

Fixing solution was then drained. Freshly prepared sensitizing solution was then poured into the tray. It was shaken on an orbital shaker for 30 minutes. After 30 minutes, sensitizing solution was drained and a sufficient amount of ddH<sub>2</sub>O was poured into the dish. It was then washed on an orbital shaker for 5 minutes and repeated 3 times.

The ddH<sub>2</sub>O was then drained. Fresh silver solution was poured into the dish and shaken on an orbital shaker for 20 minutes. The silver solution was then drained. The gels were then washed with ddH<sub>2</sub>O for one minute twice.

The ddH<sub>2</sub>O was then drained and freshly prepared developing solution was poured into tray. Developing was performed for four minutes. Developing solution was then drained and fresh stopping solution was poured into the tray. It was shaken on an orbital shaker for 10 minutes.

Finally, stopping solution was drained and ddH<sub>2</sub>O was poured into tray for washing. It was washed for 10 minutes and repeated 3 times. After washing, ddH<sub>2</sub>O was drained and fresh preserving solution was poured into dish for long term storage of the gel.

### **3.10 Analysis of 2D Gel Image**

#### **3.10.1 Materials:**

Gel Scanner from Amersham Biosciences

ImageMaster 2D v7.0 software from GE Healthcare

#### **3.10.2 Methods:**

Gel images were acquired using the gel scanner. The image was edited as necessary for orientation and size. Contrast of the gel image was then adjusted to visualize the maximum number of spots. Next, spot detection and quantification were completed automatically by using the ImageMaster program. Landmark of spots were used in spot matching to increase the rate and accuracy of matching. Subsequently, the gel images were normalized. Once the spots had been picked and matched, a statistical report was generated by the program.

### 3.11 In-gel Digestion (for Silver Staining) and Tryptic Peptide Extraction

#### 3.11.1 Materials:

Destaining solution (15 mM potassium ferricyanide(III), 50 mM sodium thiosulphate )

Reducing solution (10 mM Dithiothreitol, 40 mM Ammonium bicarbonate )

Alkylation solution (55 mM iodoacetamide, 40 mM Ammonium bicarbonate )

Washing solution (50% Acetonitrile (v/v), 40 mM Ammonium bicarbonate )

Absolute acetonitrile (ACN)

Speed vac concentrator from Thermo Scientific

Trypsin solution (7 µg/µL trypsin from Promega, 40 mM Ammonium bicarbonate )

#### 3.11.2 Methods:

Spots of interest were manually excised (1~2 mm<sup>2</sup> in size) from 4 or 5 preparative gels and pooled into a 1.5-mL microcentrifuge tube. Brown colored gel plugs were destained with 100 µL destaining solution. After destaining, proteins in gel plugs were reduced in 150 uL reducing solution for 30 minutes at 60 °C. This was then followed by alkylation using 150 uL alkylating solution for 20 minutes, incubation in the dark and at room temperature. Subsequently, the gel plugs were washed with 500 uL washing solution for 10 minutes on a mini-shaker and extensively washed for 3 times to remove any excess iodoacetamide. Fifty micro liters of ACN was used to dehydrate the gel plugs by immersing it for 15 minutes until it turned opaque white. Remaining ACN was rapidly evaporated by speed vacuum for 15 minutes. Upon drying, 25 µL of trypsin solution was added and it was incubated at 37 °C overnight. The next day, peptide rich

trypsin solution was transferred into a new 1.5-mL microcentrifuge tube. First recovery peptide from gel plugs was performed by mixing and shaking with 25 uL of 50% ACN solution for 15 minutes. An additional extraction step with 25 uL of absolute ACN was performed for 15 minutes. The peptide extraction solution was completely evaporated in a speed vacuum centrifuge. Dried peptide was stored at 4 °C until spotting on MALDI plates.

### 3.12 Spotting on MALDI Plate

#### 3.12.1 Materials:

384 - well MALDI plates

Ziptip  $\mu$ -C<sub>18</sub> (Millipore)

Trifluoroacetic acid (TFA), 0.1% (v/v) from Merck, USA

Absolute acetonitrile

Matrix  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma)

#### 3.12.2 Methods:

Ziptip was first activated using 100% acetonitrile by flushing the column bed 3 times. Organic mobile phase of the Ziptip was removed by washing and flushing with 10  $\mu$ L of trifluoroacetic acid, 0.1% (v/v) 5 times. The dried peptide mixture was reconstituted in 10  $\mu$ L of trifluoroacetic acid, 0.1% (v/v) and was then applied onto the Ziptip by flushing for 10 times to ensure that the tryptic peptides completely bind to the C<sub>18</sub> column bed. Unbound salt in the mobile phase of column was flushed using 10  $\mu$ L of trifluoroacetic acid, 0.1% (v/v). Bound tryptic peptides were eluted with 1.5  $\mu$ L elution solution. Eluted peptide mixture was then mixed with matrix  $\alpha$ -cyano-4-hydroxycinnamic acid (4-CHCA) 10 mg/mL in 1:1 ratio. Upon thoroughly mixing, matrix-peptides mixtures were spotted (7  $\mu$ L) onto 384-well MALDI plates. This was allowed to air-dry to allow for the co-crystallization of matrix and tryptic peptides. A second spotting was then performed.

### 3.13 Tandem Mass Spectrometry and Database Searching

#### 3.13.1 Equipment and software:

4800 *Plus* MALDI TOF/TOF Analyzer (Agilent Technologies, US)

GPS Explorer<sup>TM</sup> (Agilent Technologies, US)

#### 3.13.2 Methods:

All analyses and protein identification were performed using the ABI 4800 *Plus* MALDI TOF/TOF Analyzer from Applied Biosystems. Sample peptides were ionized and energized by YAG Laser shot at 355 nm wavelength. The software used for spectra acquisition was the 4000 series explorer. Minimum signal to noise ratio (S/N) was set at 25 for monoisotopic precursor selection in MS/MS. A list of acquired spectrums from MS/MS was matched with the theoretical spectrums in Swiss-PROT database. The matching task was performed using GPS Explorer<sup>TM</sup> software. Before database searching, several parameters were changed to improve the searching outcomes and speeds. The reference proteins database was confined under taxonomy *Homo sapiens*. MS/MS fragment and precursor tolerance was selected at 0.2 Da and 100 ppm respectively. Maximum peptide missed cleavage was selected at 1. Methionine oxidation and cysteine carbamidomethylation were selected as variable modifications in database searching. The search outcomes were returned as protein rank. Protein rank was determined by the probability based Mowse score. The highest ranked protein had the highest score among other candidates.



### 3.14 *In Silico* Analysis of Protein Localization and Functions

#### 3.14.1 Programs:

SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>)

SecretomeP 2.0 server (<http://www.cbs.dtu.dk/services/SecretomeP/>)

TMHMM v2.0 server (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>)

Ingenuity Pathway Analysis (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com))

Exocarta database (<http://www.exocarta.org>)

#### 3.14.2 Methods:

Canonical sequences of identified proteins were selected for all bioinformatics analysis. SignalP 4.0 server was used to predict the location of signal peptide cleavage site based on submitted identified protein amino acid sequences. The signal peptide was anticipated to be present in protein sequence if it returned D-score exceeding 0.450 (Petersen *et al.* 2011). SecretomeP 2.0 server was used to analyze which proteins were predicted to be secreted by non-classical secretion. The identified proteins were considered as non-classical secreted proteins if the N-N score was above a threshold of 0.500 (Bendtsen *et al.* 2004). TMHMM v2.0 server was used to analyze all identified proteins for transmembrane plasma protein feature. As its expected number of amino acid in transmembrane helixes (ExpAA) exceeded 18.000, it was considered as transmembrane plasma protein (Sonnhammer *et al.* 1998; Krogh *et al.* 2001; Mathivanan *et al.* 2010). Ingenuity Pathway Analysis was applied to determine the localization of identified proteins and their participation in molecular networks associated with carcinogenesis according to the well-established Ingenuity Knowledge

Base. The identified proteins were categorized as part of secretome if it possessed a signal peptide according to SignalP or it is non-classically secreted according to SecretomeP or it has transmembrane helix according to TMHMM or its localization is in the extracellular space according to Ingenuity Knowledge Base. In addition, all identified proteins were also subjected to a query search on Exocarta database to determine their presence in microvesicular bodies (Mathivanan *et al.* 2012).

### 3.15 Real-time PCR Validation

#### 3.15.1 Materials:

Primer 3 software from <http://simgene.com/Primer3>

PrimerBank from <http://pga.mgh.harvard.edu/primerbank/>

NCBI Primer-Blast from <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>

RNAqueous®-4PCR Kit from Ambion, USA

High Capacity cDNA Reverse Transcription Kit from Applied Biosystems, CA

Taqman® Gene Expression Assay from Applied Biosystems, CA

Fast SYBR Green from Applied Biosystems, CA

StepOnePlus Real-Time PCR instrument from Applied Biosystems, CA

StepOne Software v2.2 from Applied Biosystems, CA

Relative expression software tool (REST) from

<http://www.genequantification.de/rest.html>

#### 3.15.2 Methods:

Total mRNA was extracted from cell pellets using RNAqueous®-4PCR Kit. High Capacity cDNA Reverse Transcription kit was used to reverse transcribe total mRNA into cDNA templates. Owing to the high specificity and accuracy of Taqman® Gene Expression Assay, it was employed to validate differential expression of proteins of great interest - TIMP1, TIMP2 and MMP7 which mutually coordinate in a favor of cancer progression and dissemination. Fast Sybr Green was used to assay the gene

expression level of the rest of the protein candidates as a trade-off between result specificity and cost incurred. Primers were either designed using Primer3 software or retrieved from literature and PrimerBank (He *et al.* 2005; Spandidos *et al.* 2008). The specificities of targeted primers were examined using NCBI primer-Blast prior to primer purchasing. Standard curve and melt curve were plotted for all purchased primers from 1<sup>st</sup> Base Laboratory Sdn Bhd, Malaysia to ensure their efficiency and specificity. The details of primer pairs are tabulated in Table 3.3.

All PCR reactions were run using StepOnePlus Real-Time PCR instrument. The PCR was initially preheated at 95 °C for 20 s to activate *Taq* polymerase. Subsequently, DNA melting step was performed at 95 °C for 3 s followed by an annealing step at 60 °C for 30 s. A complete PCR cycle consisted of DNA melting and primer annealing stage was repeated for 40 rounds. Automatic threshold and baseline were selected to calculate Ct value. Gene expression level of different cell lines were comparatively analyzed using StepOne software v2.2. Raw data from the experiments were recalculated as a mean expression level of cancer and normal group to generalize results of comparison between two groups. Student unpaired one tailed t-test and pair wise fixed reallocation randomization test were then performed to examine significance of results from comparison among varied cell lines (Pfaffl *et al.* 2002).

**Table 3.3:** Entrez gene name of forward and reverse primer pair sequences or taqman assay ID used in real-time PCR.

No	Entrez Gene Name	NCBI RefSeq	Taqman Assay ID/ Primer pairs <sup>a)</sup>
1	Matrix metalloproteinase 7	NM_002423.3	Hs01042796_m1
2	TIMP metalloproteinase inhibitor 2	NM_003255.4	Hs00234278_m1
3	Cystatin- E/M	NM_001323.3	sense 5' - TACTTCCTGACGATGGAGATGG-3' antisense 5' - GAGTTCTGCCAGGGAACCAC-3' Primer bank ID: 325197208c1
4	Cathepsin D	NM_001909.4	sense 5'-CACCACAAGTACAACAGCGAC-3' antisense 5' - CCCGAGCCATAGTGGATGT-3' Primer bank ID: 332078524c2
5	Cadherin 2	NM_001792.3	sense 5' - AGCCAACCTTAACTGAGGAGT-3' antisense 5' - GGCAAGTTGATTGGAGGGATG-3' Primer bank ID: 215422305c2

**Table 3.3, continued**

<b>6</b>	Heterogeneous nuclear ribonucleoprotein A2	NM_002137.3	sense 5'-GAGTCCGCGATGGAGAGAGA-3'; antisense 5'-GATCCCTCATTACCACACAGTCTGT-3' Reference: (He <i>et al.</i> 2005)
<b>7</b>	Heterogeneous nuclear ribonucleoprotein B1	NM_031243.2	sense 5'-GGAGAAAACCTTTAGAAAACCTGTTTCCTTTG-3'; antisense 5'-GCTTTCCCCATTGTTTCGTAGTAGT-3' Reference:(He <i>et al.</i> 2005)
<b>8</b>	Reticulocalbin 1	NM_002901.2	sense 5'- AAGCCCACGGTGCGCAAAGA-3' antisense 5'- AGGCCTCGTGGTCGTACTGGAA-3'
<b>9</b>	Peroxiredoxin 1	NM_002574.3	sense 5'-CCACGGAGATCATTGCTTTCA-3' ; antisense 5'-AGGTGTATTGACCCATGCTAGAT-3' PrimerBank ID: 32455267b1
<b>10</b>	Proliferating cell nuclear antigen	NM_182649.1	sense 5'-ATCAACGAGGCCTGCTGGGA-3'; antisense 5'-TGGACATACTGGTGAGGTTACGC-3'

**Table 3.3, continued**

<b>11</b>	Follistatin-like 1	NM_007085.4	sense 5'-CCCAGTTGTTTGCTATCAGTCC-3' antisense 5'-TGTAGTTGCTGCCTTTAGAGAAC-3' Primer bank ID: 197304788c2
<b>12</b>	Hepatoma-derived growth factor	NM_001126050.1	sense 5'-CTCTTCCCTTACGAGGAATCCA-3'; antisense 5'-CCTTGACAGTAGGGTTGTTCTC-3' PrimerBank ID: 186928818b1
<b>13</b>	Heat shock 10kDa protein 1	NM_002157.2	sense 5'-AGTCGCTGTTGGATCGGGTTCT-3' antisense 5'-TTGGTGCCTCCATATTCTGGGAGA-3'
<b>14</b>	Laminin, beta 3	NM_001017402.1	sense 5'-GCAGCCTCACA ACTACTACAG-3'; antisense 5'-CCAGGTCTTACCGAAGTCTGA-3' PrimerBank ID: 62868216b2
<b>15</b>	Interleukin 1 receptor antagonist	NM_173842.2	sense 5'-AGAGGCCTCCGCAGTCACCTA-3' antisense 5'-TTTTCTCCCAGAGGGTTCGGCA-3'

**Table 3.3, continued**

<b>16</b>	TIMP metalloproteinase inhibitor 1	NM_003254.2	Hs00171558_m1
<b>17</b>	S100 calcium binding protein A8	NM_002964.4	sense 5'-ATGCCGTCTACAGGGATGAC-3' ; antisense 5'-ACACTCGGTCTCTAGCAATTTCT-3'  PrimerBank ID: 21614543b1
<b>18</b>	Secreted protein, acidic, cysteine-rich	NM_003118.3	sense 5'-TGAGGTATCTGTGGGAGCTAATC-3' ; antisense 5'-CCTTGCCGTGTTTGCAGTG-3'  PrimerBank ID: 48675809b1
<b>19</b>	Actin, beta*	NM_001101.3	Hs99999903_m1 /  sense 5'-CGTTACACCCTTTCTTGACAAAACC-3'  antisense 5'-GCTGTCACCTTCACCGTTCCA-3'  Reference: (He <i>et al.</i> 2005)

**Source of reference:** He, Y., Brown, M. A., Rothnagel, J. A., Saunders, N. A., & Smith, R. (2005). Roles of heterogeneous nuclear ribonucleoproteins A and B in cell proliferation. *J Cell Sci* 118(Pt 14): 3173-83.



**Footnotes of Table 3.3:**

a) Taqman gene assay ID was obtained through Applied Biosystem TaqMan<sup>®</sup> Gene Expression Assay Search. Primer pairs were retrieved from PrimerBank and literature or designed using Primer 3 software.

\* Endogenous gene control

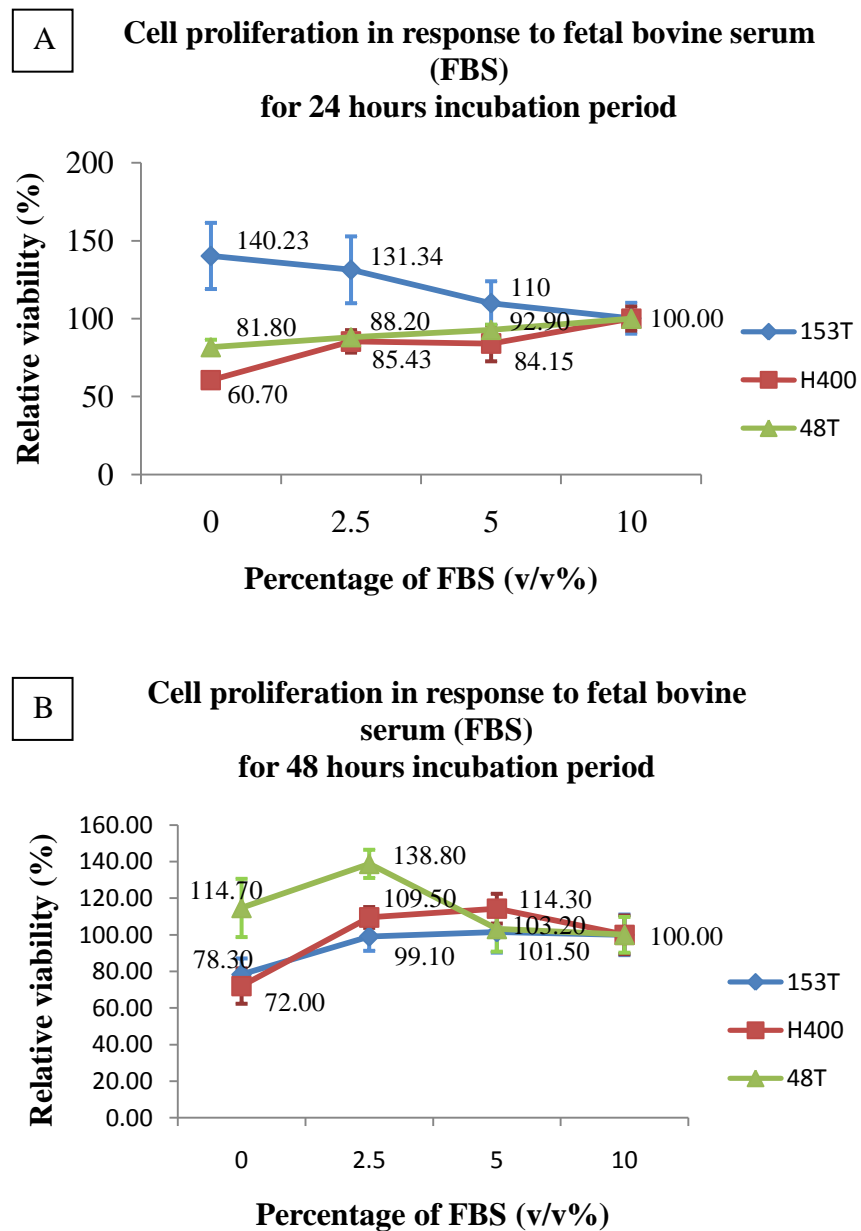
## **CHAPTER 4**

### **RESULTS**

#### 4.1 Cancer cell lines proliferation assay (MTT assay)

Cancer cell lines are usually cultured under nourishment of 10% FBS for optimum condition. However, incubation with serum-free media is a key step for secretome preparation. Under such stress environment, cell lines may proliferate slowly resulting in a different secretome profile from its original state. Therefore, impact of serum deprivation on proliferation rate of cancer cell lines with optimum protein yield was assessed by MTT assay for 24 and 48 hours. A longer incubation period of 48 hours was believed to give higher protein yield.

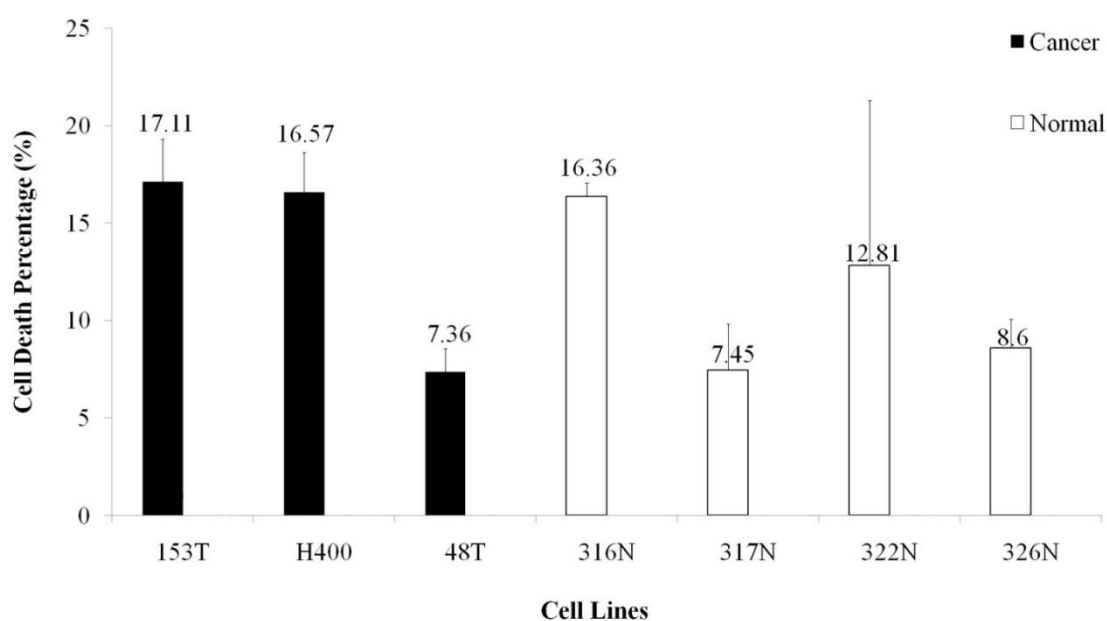
Cancer cell lines viability in 0% FBS media was significantly equal to 10% FBS furnished cancer cell lines ( $p>0.05$ ). The results also showed that for cell lines H400 and 48T, there was no significant difference in proliferation rate of 24 and 48 hours starvation periods, whereas starvation for 48 hours adversely influenced proliferation rate of 153T cell line (62% decline) compared with starvation for 24 hours. In order to avoid dramatic inhibition on proliferation of 153T cell line, an incubation period of 24 hours was determined as the optimum condition for secretome sample preparation (Figure 4.1). Normal primary cultures were not subjected to this assay because as was maintained in keratinocyte serum-free media (KSFM) optimally without serum additive.



**Figure 4.1** : Effect of fetal bovine serum concentration on cancer cell lines at different incubation period, 24 hours and 48 hours. Graph (A) and (B) are presented as mean viability  $\pm$  standard deviation. (A) cell proliferation rate of H400 and 48T were slightly inhibited after 24 hours incubation. (B) cell proliferation rate of 153T was greatly reduced after 48 hours incubation.

## 4.2 Cell death rate monitoring using Casy Ton device

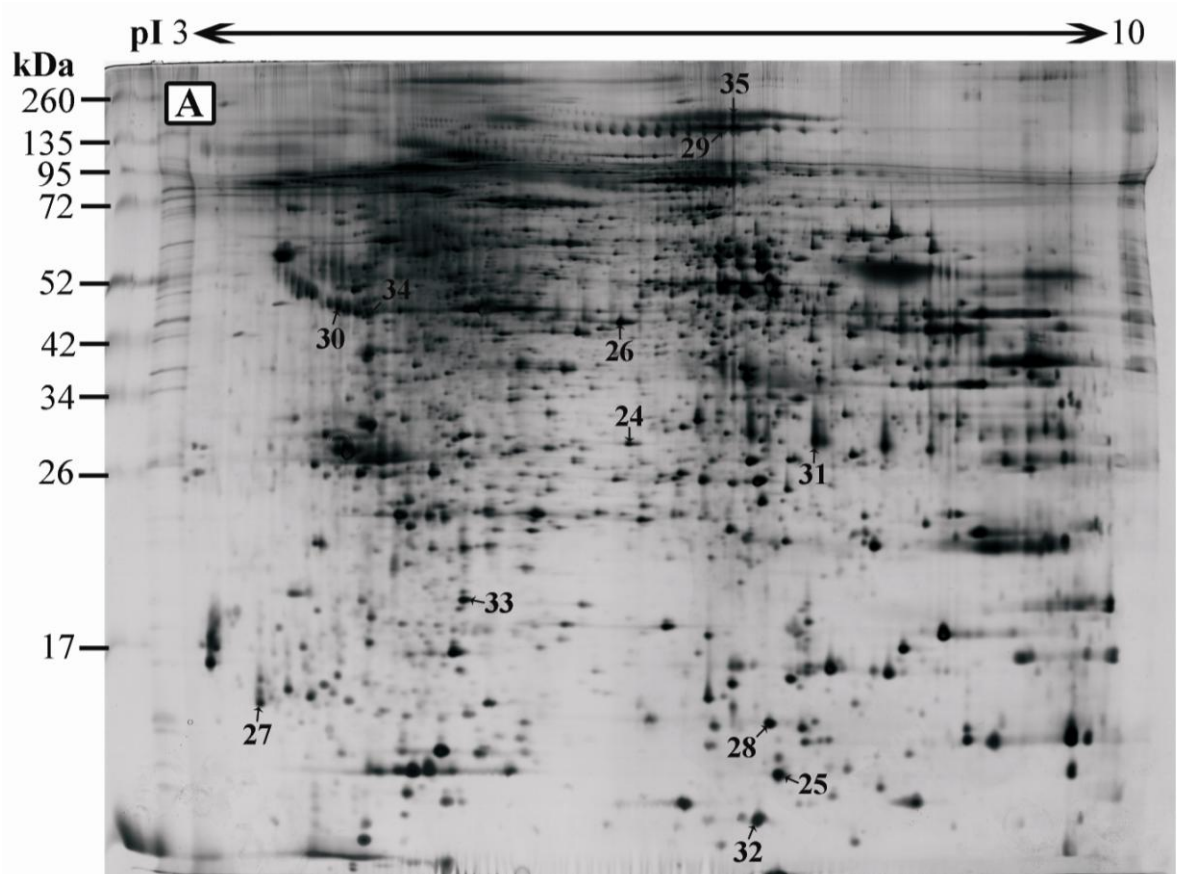
MTT-based cell viability assay was not employed to assess cell cultures death rate as it was susceptible to metabolic interference. Furthermore, total cell death percentage of cell lines could not be measured by MTT assay because reduction of tetrazolium does not happen in dead cells. Therefore, cell death of cancer cell lines and normal primary cultures were monitored using Casy® Model DT cell counter in order to achieve the least amount of cell death (< 20%, as depicted in the Figure 4.2) during secretome preparation. This was performed to minimize cytosolic protein contamination in the secretome. Cytosolic protein contamination is inevitable in secretome preparation as 24 hours of serum deprivation will inevitably induce apoptosis. Washing 5× under stringent condition with phosphate buffer saline may also cause rupture in cell membrane. However, both steps were necessary to remove abundant serum proteins which were present in the media.



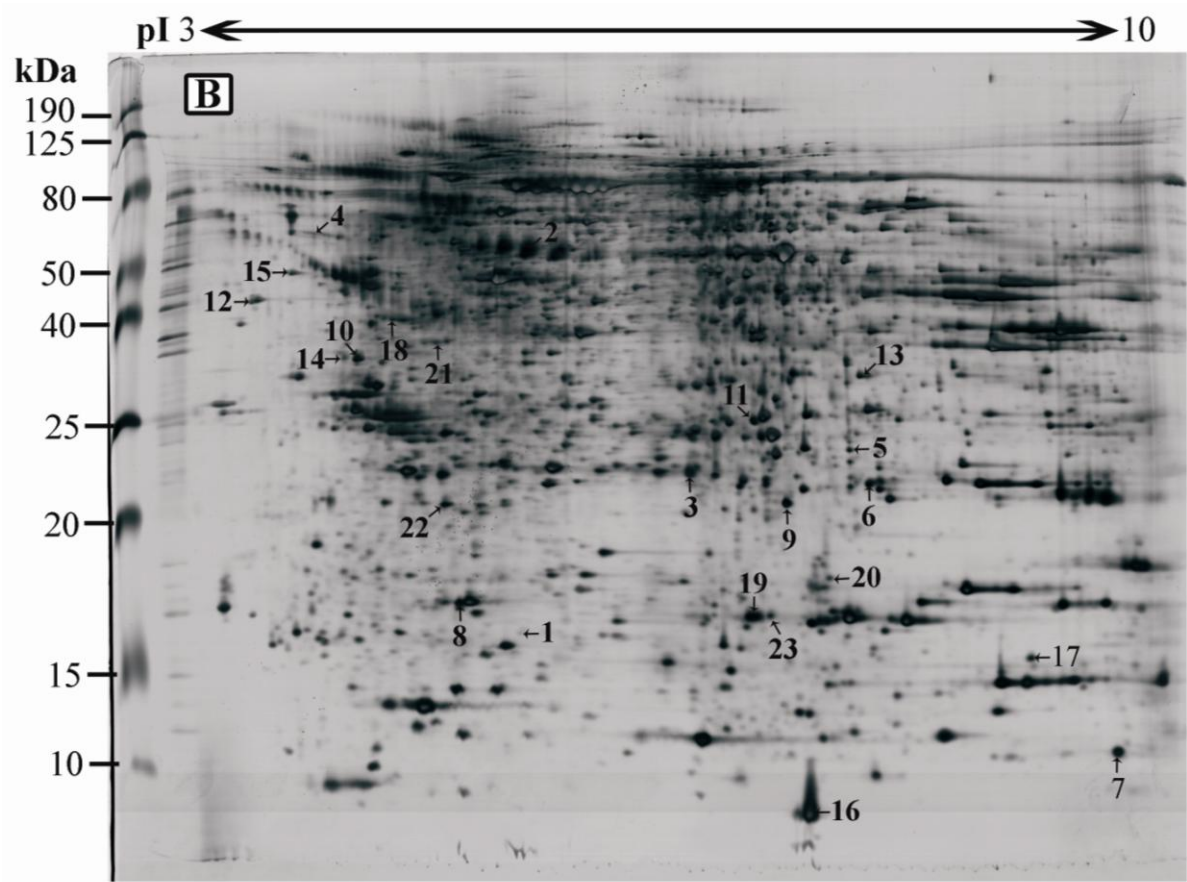
**Figure 4.2:** Cell death percentage (%) of cancer cell lines and normal primary cultures after 24 hours starvation and 5 times stringent washing with PBS. This histogram is presented as mean total cell death percentage + standard deviation. Cell death percentage of cancer cell lines was considered optimum, below 20%. Cell death percentages of normal primary culture were also considered optimum, below 20%. Low cell death percentage suggested minimal cytosolic protein contamination that may arise from cell lysis.

### 4.3 Two-dimensional (2D) Gel Images of Oral Cancer Cell Line and Normal Primary Culture

A total of 35 identified protein spots were labeled in Figure 4.3 and Figure 4.4. Their molecular weights ranged from 190 kDa to below 10 kDa and their pI values ranged from 3 to 10. The 13%T of 2D gel was good to resolve low molecular weight of secreted proteins. These proteins were separated become distinct and sharp spots. In addition, there was no protein aggregation near to the dye front. The largest 24 cm gel was used to resolve secretome for higher sample loads and better resolution.



**Figure 4.3:** A representative (20 × 24 cm) gel for normal primary culture. Identified spots were labeled with their corresponding spot ID numbers. Down-regulated proteins were labeled in this figure.



**Figure 4.4:** A representative (20 × 24 cm) gel for cancer cell lines. Identified spots were labeled with their corresponding spot ID number. Up-regulated proteins were labeled in this figure.



#### 4.4 2D Gel Analysis and Protein Spot Identification

Approximately 35 protein spots were identified with significant MOWSE score ( $p=0.05$ ). Identified spots information was shown in Table 4.1. Regulated fold changes of identified proteins were around 2-folds and above. All of the identified protein spots with ANOVA p-value and q-value less than 0.05 indicated that their differential expression was significant with less false-negative and false-positive errors.

**Table 4.1:** List of differential expressed proteins in oral cancer secretome.

Spot ID	Protein Name (Gene symbols)	Swiss-Prot Accession	Mean volume (%) $\pm$ dispersion		Fold changes <sup>(a)</sup>	ANOVA	Q-value <sup>(b)</sup>	Mowse <sup>(c)</sup>	Sequence coverage <sup>(d)</sup>
			Cancer	Normal					
1	Cadherin-2 precursor (CDH2)	P19022	0.059 $\pm$ 0.049	0.000	10 <sup>6</sup>	0.020	0.009	110	3
2	Cathepsin D (CTSD)	P07339	0.098 $\pm$ 0.092	0.000	10 <sup>6</sup>	0.038	0.016	492	47
3	Tissue inhibitor metalloproteinase-2 (TIMP2)	P16035	0.282 $\pm$ 0.182	0.005 $\pm$ 0.006	55.543	0.005	0.004	314	32
4	Follistatin like-1 (FSTL1)	Q12841	0.028 $\pm$ 0.017	0.002 $\pm$ 0.003	17.119	0.004	0.003	206	12

**Table 4.1, continued**

5	Heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2/B1)	P22626	$0.088 \pm 0.025$	$0.006 \pm 0.007$	15.231	0.000	0.000	57	7
6	Peroxiredoxin-1 (PRDX1)	Q06830	$0.073 \pm 0.061$	$0.009 \pm 0.012$	8.409	0.039	0.016	187	23
7	Heat shock 10kDa protein-1 (HSPE1)	P61604	$0.203 \pm 0.110$	$0.027 \pm 0.032$	7.614	0.004	0.003	121	40
8	Acidic nuclear phosphoprotein 32 family member B (ANP32B)	Q92688	$0.100 \pm 0.061$	$0.014 \pm 0.008$	6.993	0.008	0.005	343	23
9	Heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2/B1) , spliced variant B1	P22626	$0.146 \pm 0.069$	$0.023 \pm 0.019$	6.447	0.001	0.002	92	15
10	Proliferating cell nuclear antigen (PCNA)	P12004	$0.122 \pm 0.038$	$0.022 \pm 0.018$	5.556	0.000	0.000	193	20

**Table 4.1, continued**

11	Matrix metalloproteinase-7 (MMP7)	P09237	0.074 ±0.046	0.016 ±0.013	4.491	0.018	0.009	359	29
12	Protein SET (SET)	Q01105	0.152 ±0.055	0.034 ±0.022	4.426	0.000	0.001	378	20
13	Guanine nucleotide-binding protein subunit beta 2-like 1 (GNB2L1)	P63244	0.050 ±0.022	0.011 ±0.014	4.416	0.003	0.002	265	23
14	Proliferating cell nuclear antigen (PCNA)	P12004	0.050 ±0.019	0.012 ±0.007	4.259	0.001	0.001	125	13
15	Protein SET (SET), isoform 1/α	Q01105	0.112 ±0.033	0.027 ±0.015	4.208	0.000	0.000	239	27
16	Ubiquitin (UBB)	P62988	0.709 ±0.266	0.179 ±0.097	3.957	0.001	0.001	211	50
17	Single-stranded DNA- binding protein (SSBP1)	Q04837	0.059 ±0.031	0.018 ±0.016	3.355	0.013	0.007	279	43
18	Hepatoma-derived growth factor (HDGF)	P51858	0.099 ±0.028	0.038 ±0.005	2.575	0.000	0.000	159	27
19	Cyclophilin-A (PPIA)	P62937	0.344 ±0.093	0.156 ±0.035	2.204	0.001	0.001	439	39

**Table 4.1, continued**

20	Cofilin-1 (CFL1)	P23528	0.062 ±0.027	0.031 ±0.022	2.006	0.043	0.018	73	6
21	Elongation Factor 1-D (EEF1D)	P29692	0.050 ±0.009	0.025 ±0.004	1.998	0.000	0.000	126	28
22	Gamma glutamylcyclotransferase (GGCT)	O75223	0.111 ±0.035	0.056 ±0.022	1.978	0.006	0.005	156	25
23	Cyclophilin-A (PPIA)	P62937	0.067 ±0.028	0.036 ±0.005	1.859	0.032	0.014	116	27
24	Glutathione transferase omega-1 (GSTO1)	P78417	0.039 ±0.024	0.111 ±0.034	-2.892	0.000	0.000	64	12
25	Cystatin-M (CST6)	Q15828	0.078 ±0.032	0.238 ±0.071	-3.055	0.000	0.000	264	51
26	Leukocyte elastase inhibitor (SERPINB1)	P30740	0.048 ±0.016	0.206 ±0.061	-4.251	0.000	0.000	475	27
27	Calmodulin-like protein 3 (CALML3)	P27482	0.022 ±0.028	0.127 ±0.011	-5.716	0.000	0.000	116	11
28	Galectin-7 (LGALS7)	P47929	0.027 ±0.028	0.185 ±0.034	-6.755	0.000	0.000	534	88

**Table 4.1, continued**

29	Laminin subunit beta-3 (LAMB3)	Q13751	0.012 ±0.009	0.093 ±0.079	-7.423	0.002	0.002	653	30
30	Secreted protein acidic and rich in cysteine (SPARC)	P09486	0.018 ±0.027	0.158 ±0.082	-8.764	0.000	0.000	250	29
31	Tissue inhibitor metalloproteinase-1 (TIMP1)	P01033	0.028 ±0.041	0.405 ±0.137	-14.294	0.000	0.000	270	41
32	S100 calcium binding protein A8 (S100A8)	P05109	0.006 ±0.009	0.306 ±0.117	-48.362	0.000	0.000	122	26
33	Interleukin-1 receptor antagonist protein precursor (IL1RN)	P18510	0.000	0.116 ±0.031	-10 <sup>6</sup>	0.000	0.000	322	30
34	Reticulocalbin-1 (RCN1)	Q15293	0.000	0.030 ±0.048	-10 <sup>6</sup>	0.032	0.014	571	37
35	Laminin subunit alpha-3 (LAMA3)	Q16787	0.000	0.114 ±0.063	-10 <sup>6</sup>	0.000	0.000	122	2

**Footnotes of Table 4.1:**

- a) (-) sign represented down-regulated expression of proteins in cancer secretome. Deregulated fold changes at  $10^6$  indicated that the protein was present in cancer secretome but undetectable in normal secretome. Whereas, fold changes at  $-10^6$  indicated that the protein was present only in normal secretome but undetectable in cancer secretome.
- b) Q-value was employed to control false discovery rate (FDR) by using generated p-value list. Q-value threshold less than 0.05 was selected.
- c) Mowse score was probability based scoring scheme using by MASCOT search engine to evaluate significance of search outcomes from random matches with database. Protein with higher score above significant threshold ( $p < 0.05$ ) were recognized as identity of protein spot.
- d) Sequence coverage was a percentage of experimentally identified sequence peptides (MS/MS) against unidentified sequence peptides of same protein.

#### 4.5 Bioinformatic Analysis of Proteins Localizations

By excluding duplicated protein identities, a total of 31 proteins were subjected to *in silico* analysis for protein localizations and functions, as depicted in Table 4.2. Distinguishing “bona fide” secreted proteins from intracellular proteins which derived from dead cells is important to sort out secreted potential biomarkers. A total 24 out of 31 proteins were predicted to be secreted proteins. In other words, 77% of identified proteins were secreted via classical signal peptide dependant pathway or non-classical mechanism. However, as query search of identified proteins in the Exocarta, the non-secreted proteins that are predicted by previous programs were matching the exosomal protein candidates in the database. These non-secreted proteins may be transported out as a cargo into extracellular spaces via exosomal proteins. In summary, this study approach was reliable to investigate secreted proteome of cell lines.

**Table 4.2:** Analysis of Proteins Localization Using Different Prediction Programs and Established Databases.

Spot ID	Protein Name	Accession ID	Localization <sup>a)</sup>	SignalP <sup>b)</sup>	SecretomeP <sup>c)</sup>	TMHMM <sup>d)</sup>	ExoCarta <sup>e)</sup>
1	Cadherin-2 precursor	P19022	P	<b>0.746</b>	-	<b>23.345</b>	-
2	Cathepsin D	P07339	C	<b>0.781</b>	-	12.287	Yes
3	Tissue inhibitor metalloproteinase-2	P16035	E	<b>0.938</b>	-	0.240	Yes
4	Follistatin like-1	Q12841	E	<b>0.939</b>	-	0.803	-
5	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	N	0.104	0.081	0.033	Yes
6	Peroxiredoxin-1	Q06830	C	0.125	<b>0.528</b>	10.047	Yes
7	Heat shock 10kDa protein-1	P61604	C	0.213	<b>0.570</b>	0.000	Yes
8	Acidic nuclear phosphoprotein 32 family member B	Q92688	N	0.125	0.068	0.550	Yes



Table 4.2, continued

9	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	N	0.104	0.081	0.033	Yes
10	Proliferating cell nuclear antigen (PCNA)	P12004	N	0.139	<b>0.566</b>	0.021	Yes
11	Matrix metalloproteinase-7	P09237	E	<b>0.887</b>	-	0.538	Yes
12	Protein SET, isoform 2/ $\beta$	Q01105	N	0.112	0.106	0.001	Yes
13	Guanine nucleotide-binding protein subunit beta 2-like 1	P63244	C	0.112	0.465	0.013	Yes
14	Proliferating cell nuclear antigen (PCNA)	P12004	N	0.139	<b>0.566</b>	0.021	Yes
15	Protein SET, isoform 1/ $\alpha$	Q01105	N	0.112	0.106	0.001	Yes
16	Ubiquitin	P62988	C	0.118	<b>0.693</b>	0.000	Yes
17	Single-stranded DNA-binding protein	Q04837	C	0.116	<b>0.850</b>	0.000	Yes

Table 4.2, continued

18	Hepatoma-derived growth factor (HDGF)	P51858	E	0.120	0.477	0.004	-
19	Cyclophilin-A	P62937	C	0.107	0.339	0.001	Yes
20	Cofilin-1	P23528	N	0.102	<b>0.628</b>	0.001	Yes
21	Elongation Factor 1-D	P29692	C	0.106	<b>0.529</b>	0.003	Yes
22	Gamma glutamylcyclotransferase	O75223	C	0.108	<b>0.503</b>	0.011	Yes
23	Cyclophilin-A	P62937	C	0.107	0.339	0.001	Yes
24	Glutathione transferase omega-1	P78417	C	0.106	0.435	0.081	Yes
25	Cystatin-M	Q15828	E	<b>0.845</b>	-	16.014	-
26	Leukocyte elastase inhibitor	P30740	C	0.350	<b>0.516</b>	3.711	Yes
27	Calmodulin-like protein 3	P27482	C	0.101	<b>0.585</b>	0.000	Yes
28	Galectin-7	P47929	E	0.117	<b>0.673</b>	0.000	Yes
29	Laminin subunit beta-3	Q13751	E	<b>0.826</b>	-	0.005	Yes

**Table 4.2, continued**

30	Secreted protein acidic and rich in cysteine	P09486	E	<b>0.939</b>	-	0.007	Yes
31	Tissue inhibitor metalloproteinase-1	P01033	E	<b>0.923</b>	-	0.510	Yes
32	S100 calcium binding protein A8	P05109	C	0.100	0.280	0.043	Yes
33	Interleukin-1 receptor antagonist protein precursor	P18510	E	<b>0.726</b>	-	0.595	Yes
34	Reticulocalbin-1	Q15293	C	<b>0.848</b>	-	13.124	-
35	Laminin subunit alpha-3	Q16787	E	<b>0.630</b>	-	0.923	Yes

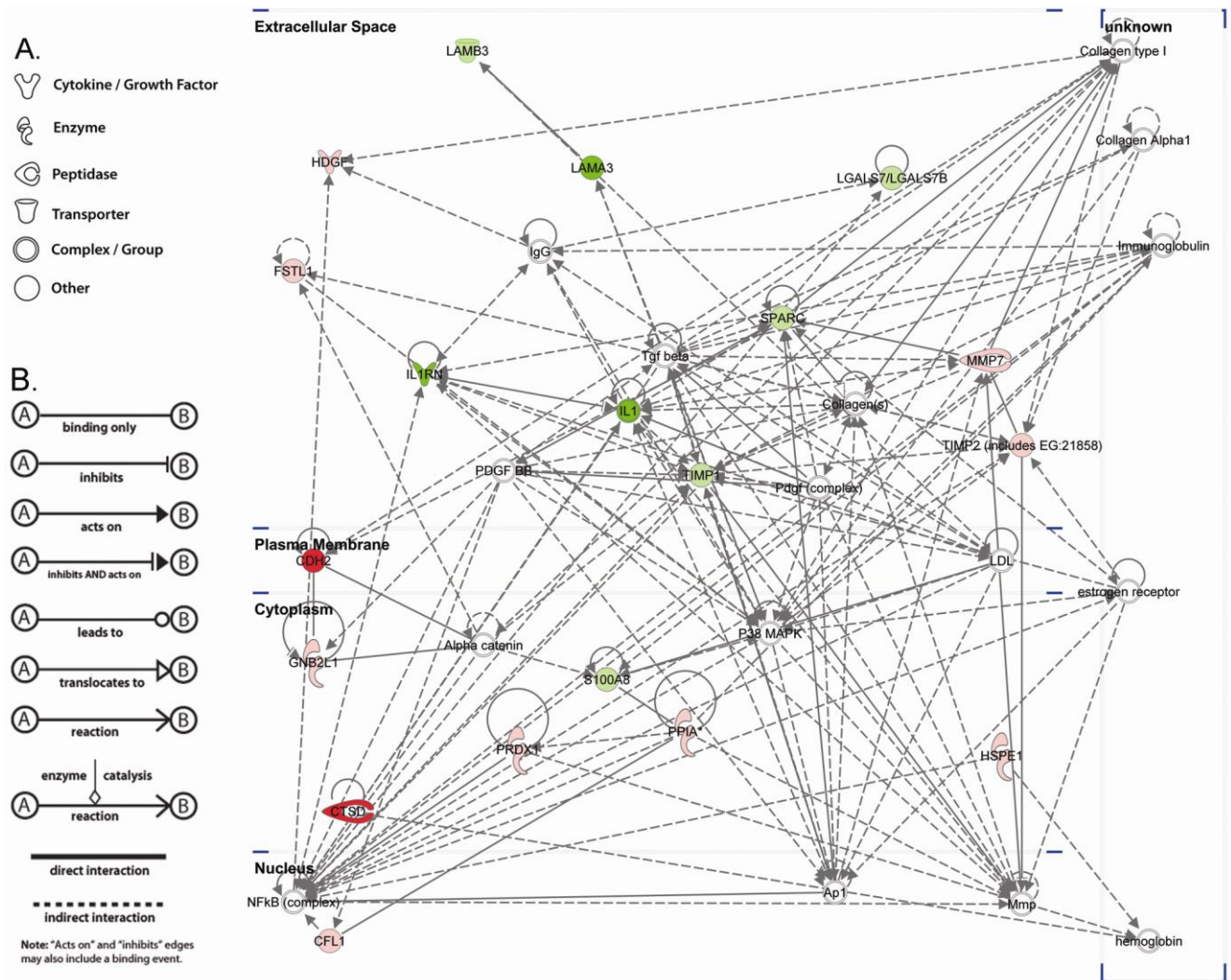
**Footnotes of Table 4.2:**

- a) Protein localization was determined according to Ingenuity Knowledge Base (IPA), where **P** denoted as plasma membrane, **C** denoted as cytoplasm, **E** denoted as extracellular space.
- b) SignalP 4.0 was employed to predict the presence of signal peptide in a sequence of protein based on a combination of several artificial neural networks. Protein was identified as classical secreted protein when its D-score exceeded 0.450. Bold number represents a value higher than D-score threshold ( $D > 0.450$ ).

- c) SecretomeP 2.0 was used to identify non-classical secreted proteins without signal peptides when its N-N score exceeded 0.500. **Number** represents a value higher than NN-score threshold ( $N-N > 0.5$ ).
- d) Transmembrane proteins have higher likelihood to be shed and secreted into extracellular space via exosome and apoptotic blebs (Sonnhammer *et al.* 1998). TMHMM v2.0 was therefore used to predict the presence of transmembrane helix in a protein. A protein was anticipated as plasma protein when its expected number of amino acid in transmembrane helixes (ExpAA) exceeded 18.000. **Number** represents a value higher than ExpAA threshold ( $ExpAA > 18.000$ ).
- e) ExoCarta, is an exosome database established based on literatures. All identified proteins were investigated based on this database. Protein was identified as an exosomal protein if it was found in the database.

#### 4.6 *In Silico* Pathway Analysis of Protein Molecules

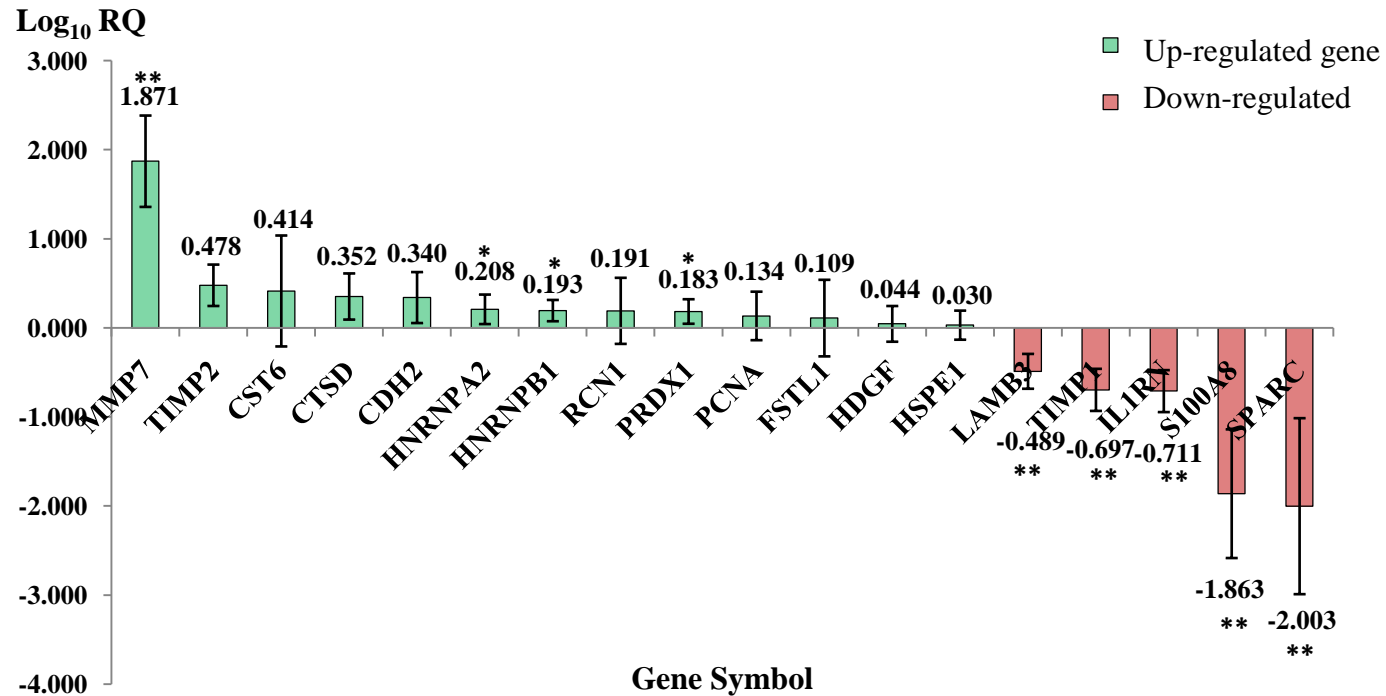
Based on the IPA core analysis, the list of identified proteins was highly relevant in cancer progression. These proteins were likely involved in tumor growth, metastasis, hyperproliferation, tumorigenesis, neoplasia, hyperplasia, and cell transformation. A top scored molecular network as depicted in Figure 4.5 in IPA core analysis was participated by these identified proteins that are LAMB3, LAMA3, MMP7, LGALS7, HDGF, FSTL1, SPARC, IL1RN, TIMP1, TIMP2, CDH2, GNB2L1, S100A8, PRDX1, PPIA, CTSD, HSPE1, and CFL1. These identified proteins are also interrelated with oncoproteins in the molecular network such as NF-K $\beta$ , TGF- $\beta$ ,  $\alpha$ -catenin, AP-1, p38 MAPK, PDGF, MMPs, estrogen receptor and IL-1. According Ingenuity Knowledge Base, expression dynamics of these proteins is highly relevant to cancer development and cell motility.



**Figure 4.5:** A top-scored molecular network with 18 identified proteins implicated in cancer progression according to IPA (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)). All protein molecules in the network were represented as gene symbol labeled nodes. The biological relationship between two nodes is represented as an edge. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product. A) molecule types, B) relationship types.

#### 4.7 Real Time PCR Results Analysis

Although protein expression was discrepant with messenger RNA expression level due to protein post-modification or proteolytic cleavage, real-time PCR is still remain robust and rapid as preliminary validation. The identified proteins were subjected to qPCR validation only if they are plausible secreted proteins or higher expression fold-change proteins or closely cancer-associated proteins. The results, which are shown in Table 4.3 and Figure 4.6, demonstrated that the direction of transcript level regulation of all selected proteins was parallel to their protein expression except CST6 and RCN. Small differences between efficiency corrected RQ and  $\Delta\Delta C_T$  based RQ of all genes showed that the effect of inequality of amplification efficiency was negligible. Some of selected candidates showed insignificant differences ( $p > 0.05$ ) of transcript expression level between cancer group (48T, 153T, H400) and normal group (316N, 317N, 322N, 326N) due to higher variation among cell lines in a group. In summary, significant differentially expressed genes with student's t-test  $\leq 0.05$  were MMP7, hnRNP A2, hnRNP B1, PRDX1, LAMB3, TIMP1, IL1RN, S100A8, and SPARC. While, significantly differential expressed genes with pair-wise randomization test  $\leq 0.05$  were MMP7, LAMB3, TIMP1, IL1RN, S100A8, and SPARC only. All significantly dysregulated genes were regulated in a fold change range from ~1.5 folds to as high as ~100 folds.



**Figure 4.6:** Base ten Logarithm relative quantity (Log<sub>10</sub>RQ) of targeted genes in cancer cell lines were measured using qPCR. This histogram is presented as mean Log<sub>10</sub>RQ ± error bars. Error bars indicate Log<sub>10</sub>RQ<sub>min</sub> and Log<sub>10</sub>RQ<sub>max</sub>. Two asterisks (\*\*) indicates that statically significant of differential gene expression by both student t-test (p = 0.05) and pair wise fixed reallocation randomization test (p = 0.05). Single asterisk (\*) shows that differential gene expression is statically significant by either student t-test or randomization test only.



**Table 4.3:** Differential expression of targeted genes between normal and oral cancer cell lines and their significance was tested with student's t-test and randomization test.

No	Entrez Gene Name	Gene Symbol	NCBI RefSeq	RQ	Fold change <sup>a)</sup>	Efficiency Corrected RQ <sup>b)</sup>	Log <sub>10</sub> RQ <sup>c)</sup>	RQ Min <sup>d)</sup>	RQ Max <sup>d)</sup>	t-test <sup>e)</sup>	REST p-value <sup>f)</sup>
1	Matrix metalloproteinase 7	MMP7	NM_002423.3	74.254	74.254	74.254	1.871	22.790	241.931	<b>0.007</b>	<b>0.029</b>
2	TIMP metalloproteinase inhibitor 2	TIMP2	NM_003255.4	3.004	3.004	3.004	0.478	1.759	5.131	0.113	0.190
3	Cystatin- E/M	CST6	NM_001323.3	2.592	2.592	2.416	0.414	0.618	10.879	0.391	0.381
4	Cathepsin D	CTSD	NM_001909.4	2.247	2.247	2.262	0.352	1.237	4.082	0.092	0.328
5	Cadherin 2	CDH2	NM_001792.3	2.187	2.187	2.150	0.340	1.132	4.225	0.237	0.433

Table 4.3, continued

6	Heterogeneous nuclear ribonucleoprotein A2	HNRNP A2	NM_002137.3	1.614	1.614	1.563	0.208	1.102	2.363	<b>0.007</b>	0.214
7	Heterogeneous nuclear ribonucleoprotein B1	HNRNP B1	NM_031243.2	1.561	1.561	1.516	0.193	1.186	2.055	<b>0.030</b>	0.110
8	Reticulocalbin 1	RCN1	NM_002901.2	1.551	1.551	1.501	0.191	0.660	3.643	0.258	0.581
9	Peroxiredoxin 1	PRDX1	NM_002574.3	1.525	1.525	1.483	0.183	1.111	2.094	<b>0.029</b>	0.197
10	Proliferating cell nuclear antigen	PCNA	NM_182649.1	1.360	1.360	1.363	0.134	0.727	2.545	0.253	0.799
11	Follistatin-like 1	FSTL1	NM_007085.4	1.286	1.286	1.268	0.109	0.478	3.460	0.397	0.770
12	Hepatoma-derived growth factor	HDGF	NM_001126050.1	1.108	1.108	1.100	0.044	0.698	1.758	0.314	0.817
13	Heat shock 10kDa protein 1	HSPE1	NM_002157.2	1.071	1.071	1.069	0.030	0.736	1.560	0.376	0.862

Table 4.3, continued

14	Laminin, beta 3	LAMB3	NM_001017402.1	0.325	-3.080	0.346	-0.489	0.207	0.509	<b>0.007</b>	<b>0.050</b>
15	TIMP metalloproteinase inhibitor 1	TIMP1	NM_003254.2	0.201	-4.975	0.201	-0.697	0.117	0.346	<b>0.016</b>	<b>0.001</b>
16	Interleukin 1 receptor antagonist	IL1RN	NM_173842.2	0.195	-5.137	0.208	-0.711	0.113	0.335	<b>0.011</b>	<b>0.052</b>
17	S100 calcium binding protein A8	S100A8	NM_002964.4	0.014	-72.892	0.018	-1.863	0.003	0.072	<b>0.015</b>	<b>0.026</b>
18	Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	NM_003118.3	0.010	-100.640	0.013	-2.003	0.001	0.097	<b>0.041</b>	<b>0.049</b>

**Footnotes of Table 4.3:**

- a) Fold changes was derived from Relative quantity (RQ) by  $\frac{RQ}{1}$  if  $RQ > 1$  or  $-\frac{1}{RQ}$  if  $RQ < 1$ . (-) sign represents down-regulated expression of mRNA copies in cancer cell lines.
- b) Efficiency corrected RQ is adjusted RQ according to efficiency of amplification under the optimal PCR conditions. It is used to assess the effect of inequality of amplification efficiency between targeted gene and endogenous control on the expression level of gene transcript. If efficiency corrected RQ is closer to RQ, which was calculated based on comparative  $\Delta\Delta C_t$ , the effect of inequality of amplification efficiency will be considered small and negligible.
- c) Base ten logarithms RQ shows regulated direction of gene expression with relatively smaller numerical value.
- d) The RQ minimum and RQ maximum define statistical boundaries for true RQ value to fall within the RQ Min/Max range with a 95% confidence interval. The RQ Min/Max is calculated using the equation:

$$2^{-(\Delta\Delta C_{T(s,t)} \pm T \times VAB(C_{T(s,t)}))}$$

where  $\Delta\Delta C_{T(s,t)} = \Delta C_{T(s,t)} - \Delta C_{T(\text{calibrator},t)}$ ; s=sample name; t=target detector; T=student's T value at the selected confidence setting using a degree of freedom that is associated with the test sample  $\Delta C_{T(s,t)}$ ; and VAB is the Applied Biosystems' variability function for calculating the variability of the test sample  $\Delta C_T$  statistic.

- e) Student t-test (one-tailed, unpaired, equal variance) was performed to hypothetically test significance of differences between two groups. It is powerful and higher sensitive test when two groups' data points are normally distributed. A p-value cutoff 0.05 was selected. P-values less than or equal to 0.05 are bold.
- f) Relative expression software tool (REST) developed by Michael W. Pfaffl et al (Pfaffl *et al.* 2002) was used to perform pair wise fixed reallocation randomization test without making any assumptions about distribution while remaining as powerful as other standard tests. A p-value cutoff 0.05 was selected. P-values less than or equal to 0.05 are bold.

## **CHAPTER 5**

### **DISCUSSIONS**

## 5.1 2DE Proteome Analysis and Protein Identification

A total of 35 protein spots with significantly changed abundance ( $p < 0.05$  and  $q < 0.05$ ) were identified by tandem mass spectrometry. Their deregulated fold changes were ranging from around 2 to as high as  $10^6$ . These identified proteins were subsequently subjected to bioinformatics.

## 5.2 Bioinformatics Analysis of Identified Proteins Localizations and Functions

Cancer cell secretome encompasses a variety of secreted proteins which may aid in carcinogenesis. They can be secreted via the signal peptide dependent pathway or the non-classical pathway or exosomal bodies. In signal peptide dependent pathway, precursor proteins which possess N-terminal signal peptides are directed to endoplasmic reticulum (ER). They are subsequently transported to the golgi apparatus for post-modification and re-assortment. Ultimately, they are transported to the plasma membrane surface in golgi-derived vesicles and liberated into the extracellular spaces via exocytosis. This pathway is termed as the classical secretory pathway. However, some secreted proteins without signal peptides are secreted through a novel flip-flop mechanism and direct translocation into the extracellular region (Karagiannis *et al.* 2010). In addition, they can also be secreted via exosomes. Exosomal bodies are generated by inward budding of the endosomal membrane within large multivesicular bodies (MVBs). The fusion of MVBs with the cellular plasma membrane causes the liberation of inner exosomal vesicular bodies into the extracellular space (Mathivanan *et al.* 2010). All golgi independent secretory pathway are termed as non-classical secretory pathway. Furthermore, shedding of the ectodomain of transmembrane proteins may also contribute to the cancer secretome. Therefore, understanding the secretory pathway of

the identified proteins or their localization would help us to sort out “bona fide” secreted proteins from the identified proteins for further analysis.

Thirty-one unduplicated proteins were subjected to *in silico* analysis to predict their localization using signalP 4.0, secretomeP 2.0, TMHMM, Ingenuity Knowledge Base, and Exocarta database. Based on the *in silico* analysis, 12 proteins were predicted to possess N-terminal signal peptides and 11 proteins without signal peptides were secreted non-classically. Only one protein was predicted to be a trans-membrane protein. Based on the Ingenuity Knowledge Base and Exocarta database searching, 11 proteins were recognized as extracellular proteins and 26 proteins were secreted via exosomes. In conclusion, all the identified proteins were predicted to be secreted proteins.

Expression dynamics of the identified proteins were also subjected to Ingenuity Pathway Analysis (IPA) for pathway analysis. A top-scored molecular network (refer to Figure 4.5) was generated based on the Ingenuity Knowledge Base. This molecular network was closely associated with carcinogenesis and was predicted to mediate other recognized oncogenic proteins within the molecular network. Identified proteins that were located in the extracellular space of the molecular network were LAMB3, LAMA3, LGALS7, HDGF, FSTL1, IL1RN, IL1, SPARC, MMP7, TIMP2, and TIMP1. These secreted proteins have higher potential to be oral cancer biomarker candidates.

### **5.3 Transcript Expression Analysis of Selected Altered Proteins**

Proteins that were potentially secreted, have potential role(s) in carcinogenesis and have the greatest change in abundance were selected for RNA transcript expression analysis. Although RNA levels do not always correlate with protein levels, it does



provide additional information with regards to whether the expression of the protein concerned is regulated at the transcript level.

During PCR, amount of a gene is theoretically increased twice with each successive PCR cycle. In fact, the number of gene is never double exactly in a real PCR run due to imperfect amplification efficiencies. Imperfect amplification efficiencies is caused by non-optimal PCR running conditions such as  $T_m$  of a primer pair, length of the amplicon, GC/AT composition of a primer pair, primer pair concentration, template concentration, concentration of  $Mg^{2+}$ , and sample contaminants. The condition may vary from one targeted gene sequence to another. In a comparative real time PCR experiment, multiple genes and endogenous control genes are run under the same condition. This condition is a general optimum condition for all genes but not specific for each gene. This may lead to unequal amplification efficiency for all genes. In a comparative genes expression analysis, targeted gene expression is calculated by normalized with housekeeping gene expression. Unequal amplification efficiency between them may leads to inaccurate relative gene expression calculation results. Therefore, a RQ corrected calculation approach was introduced in this experiment to examine the effect of inequality of amplification efficiency. The results indicated that such effect is negligible for all targeted genes and  $\Delta\Delta C_t$  based RQ were reliable to reflect their true gene differential expression level.

The student's t-test of qPCR results with assumption of normal sample distribution showed that MMP7, hnRNP A2, hnRNP B1, PRDX1, LAMB3, TIMP1, IL1RN, S100A8, and SPARC were significantly deregulated. Without any assumption of sample distribution pattern, pair wise randomization test showed that only MMP7, LAMB3, TIMP1, IL1RN, S100A8, and SPARC transcript were significantly

deregulated. However, the student's t-test is much more sensitive than the randomization test if the sample distribution is normal. It can however, be a valuable reference for short listing potential biomarkers candidates. In addition, these proteins were implicated in the top-scored molecular network generated by Ingenuity Knowledge Base with the exception of hnRNP A2/B1. These proteins with other known cancer associated proteins are reviewed in the following section.

#### 5.4 Identified Proteins Known to Have Roles in Carcinogenesis

Matrix Metalloproteinase VII (MMP-7) and its natural inhibitors, TIMP-1 and TIMP-2 have roles in cancer dissemination. Up-regulation of MMPs accompanied by down-regulation of their natural inhibitors is the common expression pattern in malignant tumors (Egeblad and Werb 2002). The relative expression of MMP-7 and TIMP-1 has also been demonstrated in a study of non-small cell lung cancer (NSCLC) (Safranek *et al.* 2007).

MMP-7 also known as matrilysin which is a member of the MMP family proteases was found to be increased in the oral cancer secretome. Its molecular weight was estimated to be above 25 kDa (Figure 4.4, spot ID 11) and part of the propeptide sequence RFYLYDSETK, and FFGLPITGMLNSRVIEIMQKPRCGVPDVAE were also identified (refer to Appendix A, spot ID. 11). Thus, it was likely to be the latent form of MMP-7 or pro-MMP-7 (MW 28 kDa, its active form is 19 kDa). Both latent and active forms of MMP-7 have been demonstrated to be implicated in colorectal cancer patients who had liver metastases (Zeng *et al.* 2002). Substrates for MMP-7 are commonly implicated in oral carcinogenesis and these include CD95/Fas receptor, Fas Ligand (FASL), E-cadherin, and heparin-binding epidermal growth factor (Fingleton

2006). By mediating these substrate molecules, MMP-7 is able to regulate cancer cell apoptosis, angiogenesis, invasion and metastasis. Studies have demonstrated that CD95 ligand cleavage product of MMP-7 inhibits apoptosis induced by chemotherapeutic drugs and breakdown of Fas receptor by MMP-7 reduces apoptosis sensitivity of HepG2 cells (Mitsiades *et al.* 2001; Strand *et al.* 2004). Moreover, it enables the proteolytic release of membrane-anchored HB-EGF which is a potent proangiogenic factor thus promoting cell survival and angiogenesis (Handsley and Edwards 2005). With a function of protease, it cleaves E-cadherin which is involved in cell-cell adhesion to promote cancer cell invasion and trigger the EMT (Egeblad and Werb 2002). As a result, MMP-7 is able to perform multiple functions to facilitate cancer metastasis.

Over-expression of MMP-7 has been reported in a wide variety of cancers particularly those of epithelial origin. Miyata *et al.* reported that overexpression of MMP-7 was a strong prognostic marker for poor prognosis in patients with renal cell carcinoma (Miyata *et al.* 2006). Gene expression of MMP-7 was observed to be highly up-regulated in the early stage of oral cancer especially the G allele type of MMP-7 gene (Vairaktaris *et al.* 2007). Furthermore, a finding demonstrated that over-expression of MMP-7 was common in more aggressive OSCC tissues but absent in mild verrucous carcinoma tissues (Impola *et al.* 2004). Real time PCR results indicated that the transcript level of MMP-7 was significantly up-regulated for 74-fold. Thus, MMP-7 is a good candidate for further investigation as a potential biomarker candidate or a therapeutic target. Its potential as a serum biomarker has been substantiated in colorectal cancer patients (Maurel *et al.* 2007). Several MMP inhibitors are emerging as a new regime for cancer therapy such as Marimastat which can inhibit a broad spectrum of MMPs including MMP-7 and showed a good survival benefit in clinical trials (Hidalgo and Eckhardt 2001; Overall and Lopez-Otin 2002).

In contrast to the expression of MMP-7, tissue inhibitor metalloproteinase I (TIMP-1) was found significantly down-regulated at the mRNA and protein expression level in oral cancer cell lines. TIMP-1 has a molecular weight of 28 kDa and 2 N-glycosylated sites. It can bind to all MMPs reversibly but poorly inhibits MMP-19 and several types of membrane-bound MMPs (Baker *et al.* 2002). Down-regulation of TIMP-1 leads to unlocking of MMPs activities which facilitates cancer invasion by degradation of the ECM components (Egeblad and Werb 2002). Furthermore, TIMP-1 down-regulation reduces contact inhibition of cancer growths as well as confers cancer cell lines with resistance towards apoptosis (Hojilla *et al.* 2003).

Tissue inhibitor of metalloproteinase II (TIMP-2) was significantly increased in cancer secretome. However, its transcript level showed insignificant up-regulation. TIMP-2 exhibits dual functions in the MMPs inhibition as well as in the activation of matrix metalloproteinase II (MMP-2) which is essential for breaking down of the ECM and promoting carcinoma invasion to surrounding tissues (Lu *et al.* 2004). Over-expression of TIMP-2 is also associated with poor prognosis in invasive bladder cancer (Grignon *et al.* 1996). Furthermore, TIMP-2 has been substantiated as a valuable prognostic marker in tumor staging of colorectal cancer (Ring *et al.* 1997). An immunohistochemical study of 30 random tumor tissue samples by Qu *et al.* demonstrated that association of TIMP-2 up-regulation with oral squamous cell carcinoma (Qu *et al.* 2006).

Besides up-regulation of proteases of the MMP family, a protease of the cathepsin family, Cathepsin-D was increase in oral cancer cell line secretome but was not significantly different in expression at the transcript level. The 52 kDa pro-cathepsin-D was identified in this study (Figure 4.4, spot ID 2). Secreted pro-cathepsin-

D can be endocytosed by cancer cells again or by adjacent fibroblasts. It exhibits optimum enzyme activity under acidic conditions. The roles of cathepsin-D in carcinogenesis are in facilitating metastasis, proliferation and angiogenesis. Because of its activation is only accomplished under an acidic environment, it is believed that cathepsin-D is devoid of proteolytic functions and rather acts as a secreted binding protein during tumorigenesis (Liaudet-Coopman *et al.* 2006). In a comparative analysis between immunohistochemistry and routine histopathology, intense staining of cathepsin-D was apparent in oral SCC (Yogesh *et al.* 2011). Moreover, cathepsin-D was also shown to be a potential serum marker in glioma patients and its up-regulation was associated with poor prognosis of gliomas (Fukuda *et al.* 2005).

Cell dissociation is a key step towards cancer cell invasion and metastasis. Components of the ECM - collagens, glycoproteins(eg. Laminin), and proteoglycans - form a scaffold to support cells attachment. However, it hinders cell dissociation and migration (Egeblad and Werb 2002). Disruption of this structure is necessary for the movement of cancer cells. Our results indicated that laminin subunit beta-3 and alpha-3 were found to be decreased in abundance in the oral cancer secretome suggesting lower secretion or breakdown of laminin 5 (Ln-5) in cancer cell lines. Transcript levels of laminin subunit  $\beta 3$  were also found to be significantly down-regulated. Ln-5 is composed of 3 subchains, alpha-3, beta-3, and gamma-2 forming a heterotrimeric protein. It plays a role in the formation of the hemidesmosome and helps to attach normal basal cells to the basal lamina (Hao *et al.* 1996). Suppression of Ln-5 subunit  $\gamma$ -2 in non-invasive OSCC cell lines indicated that subunit  $\gamma$ -2 modulated expression of subunit  $\alpha/\beta$ -3 and augmented cancer cells motility and invasion (Yuen *et al.* 2005). Furthermore, significant down-regulation of Ln-5 has been reported in breast carcinoma

and prostate cancer (Hao *et al.* 1996; Martin *et al.* 1998). Hence, breakdown of this barrier will likely enhance cancer cell migration and invasion to a distant site.

By contrast, a classical type of extracellular matrix adhesion molecule N-cadherin was up-regulated in oral cancer cell lines secretome. A cleaved fragment of N-cadherin which had an estimated molecular weight of 15 kDa ~ 20 kDa was detected in the secretome. A switching from E-cadherin to N-cadherin has been found in carcinoma cell lines and is responsible for epithelial to mesenchymal transition (Hajra and Fearon 2002). The switching of E-cadherin to N-cadherin has also been shown to be manifested in an immunohistochemical study of oral SCC tissues which showed a greater down-regulation of E-cadherin in the positive N-cadherin group (Pyo *et al.* 2007). Moreover, Diamond, *et al* demonstrated that up-regulation of N-cadherin in OSCC cell lines facilitated cancer cell motility. They also revealed that loss of regulatory mechanism of TGF- $\beta$ 1 which mediated N-cadherin expression led to malignant transformation of the oral epithelium (Diamond *et al.* 2008). As a result, N-cadherin overexpression may be a crucial element for EMT progression of oral cancer cells.

Increased abundance of heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) and their corresponding transcripts was observed. Protein hnRNP A2/B1 (Figure 4.4, spot ID. 9) was determined as the B1 spliced variant due to the unique B1 variants amino acid sequence, TLETVPLER was identified in MS/MS analysis (refer to Appendix A, spot ID. 9). However, the variant of spot ID. 5 was not determined. Two identified hnRNP A2/B1 spots have lower molecular mass than the theoretical mass of A2 (34 kDa) or B1 (37 kDa) variants. A2/B1 pre-mRNA has been reported to undergo alternative splicing to produce two novel isoforms known as B1b (which exclude exon 9) and A2b (which exclude exon 2 and 9) resulting in the

production of smaller isoforms. Losing exon 9 which encodes part of the glycine rich domain (GRD) of hnRNP A2/B1 impairs M9 nuclear localization signal, A2b and B1b are therefore no longer restricted to the nucleolus and are able to be easily trafficked into the cytoplasm (Pollard *et al.* 1996; Han *et al.* 2010). Furthermore, all MS/MS-identified peptide sequences of hnRNP A2/B1 lie within the N-terminal region and are devoid of identified peptides in GRD. Therefore, it was plausible that these novel spliced variants of hnRNP A2/B1 in the oral cancer secretome were likely devoid of GRD as well.

Heterogeneous nuclear ribonucleoproteins A2/B1 is a telomeric-bound protein which functions in recruiting the enzyme telomerase for maintenance of telomere length and thus preventing tumor from entering the senescence phase (Carpenter *et al.* 2006). Hence, it is believed that hnRNP A2/B1 endows cancer cells with eternal life. Furthermore, RNAi suppression of hnRNP A2 variant expression in cancer cells has been shown to lead to a non-apoptotic pattern of slow-down in cell proliferation. It indicated that these hnRNP proteins play a role in cell proliferation (He *et al.* 2005). In addition, several studies showed that both hnRNP proteins were highly up-regulated in human lung cancer (Sueoka *et al.* 1999; Pino *et al.* 2003; Wu *et al.* 2003). Thus, over-expression of hnRNP A2/B1 is believed to play an important role in oral cancer cells unlimited proliferation.

Galectin-7 (Gal-7) and leukocyte elastase inhibitor (LEI) have been implicated in programmed cell death or apoptosis. Both were found to be significantly reduced in the oral cancer secretome. Down-regulation of these proteins may confer cancer cells with anti-apoptotic properties. Apoptosis induced by p53 has been studied by Polyak *et al.* They showed that Gal-7 was one of the highly expressed primary transcripts in

transfected p53-expressing cells (Polyak *et al.* 1997). Gal-7 overexpression has also been observed in UV-induced apoptotic keratinocytes implying that Gal-7 is associated with the apoptosis process (Bernerd *et al.* 1999). Proapoptotic functions of Gal-7 are triggered through JNK activation and liberation of mitochondrial cytochrome c (Kuwabara *et al.* 2002). Tumor growth suppression effect has been demonstrated in a study showing Gal-7-transfected cells growing slower than normal cells *in vitro* with a marked reduction of tumor formation of Gal-7-transfected cells in severe combined immunodeficient mice (Ueda *et al.* 2004). Another apoptosis-related protein, LEI from porcine spleen has been investigated by Torriglia *et al.* They demonstrated that LEI underwent post-translational modification and transformed into L-DNase II which is activated during apoptosis (Torrighia *et al.* 1998). As a result, oral cancer cells may be able to escape from p53-induced apoptosis by down-regulation of Gal-7 and LEI.

Peroxiredoxin 1 protein and its transcripts were significantly increased in oral cancer secretome. Peroxiredoxin 1 acts as a peroxide scavenger and protein chaperone intracellularly. Its fundamental role as a peroxidase shield cancer cells from peroxide damage induced by apoptosis but not by other chemotherapeutic agents (Berggren *et al.* 2001). Overexpression of peroxiredoxin 1 has been shown to promote prostate tumor growth and increased levels of angiogenic proteins within the tumor microenvironment. Additionally, it can also stimulate endothelial cell proliferation, migration, and differentiation in a toll-like receptor 4- and VEGF-dependent manner (Riddell *et al.* 2011). Extracellular peroxiredoxin 1 has been demonstrated to stimulate TLR4-dependent secretion of TNF- $\alpha$  and IL-6 which are proinflammatory cytokines implicated in the activation of NF- $\kappa$ B in tumor cells (Riddell *et al.* 2010). Overexpression of peroxiredoxin 1 has also been detected in lung cancer and oral cancer



specimens and its potential prognostic values were evident (Yanagawa *et al.* 2000; Kim *et al.* 2008).

Proliferating cell nuclear antigen (PCNA) was increased in the oral cancer secretome but was not significantly different at the transcript level. PCNA is an essential component in DNA replication and cell proliferation. In a study of 114 cases at the University of Malaya (UM), Zain *et al.* demonstrated that cancerous tissues had a higher percentage of PCNA positive nuclei (R.B.Zain *et al.* 1995). A 36 kDa acidic form of PCNA has been reported to be specifically associated with aggressive breast tumors. This acidic cancer associated form of PCNA (caPCNA) was resulted from a unique posttranslational modification in cancer cells instead of random genetic mutation events (Bechtel *et al.* 1998). A caPCNA antibody has been developed to study its potential value as a biomarker (Malkas *et al.* 2006). However, a current attempt to detect caPCNA in sera using ELISA showed that differences between control and cancer groups were insignificant (Swaby 2010). Nevertheless, this acidic form of PCNA was also detected in this study (Figure 4.4, spot ID 10 & 14). However, differential gene expression of PCNA was found to be insignificant and this may attributed by its unique posttranslational modification.

A cancer cell growth promoting agent, hepatoma-derived growth factor (HDGF) was also significantly increased in the secretome. HDGF stimulates the proliferation of several types of cells such as fibroblasts and endothelial cells and is involved in the early stages of organ grow. Its growth promoting properties in lung cancer cells have been extensively studied and indicated that shRNA knock-down expression of HDGF suppressed anchorage-independent growth and invasion of lung cancer cells (Zhang *et al.* 2006; Meng *et al.* 2010). The prognostic significance of HDGF has also been

explored in pancreatic cancer and early-stage non-small-cell lung cancer (Ren *et al.* 2004; Uyama *et al.* 2006). A more recent study on HDGF expression in oral cancer cells demonstrated that HDGF augmented cancer cell proliferation and high nuclear HDGF expression was correlated with tumor stage and poor prognostic outcome (Lin *et al.* 2012).

A hydrolyzing enzyme, gamma glutamyl cyclotransferase (GGCT) was found to be increased in the oral cancer secretome. GGCT has been implicated in glutathione homeostasis where it hydrolyzes the dipeptide  $\gamma$ -glutamylcysteine into 5-oxoproline and cysteine amino acid (Oakley *et al.* 2008). It has also been shown to be involved in cancer proliferation and invasion (Uejima *et al.* 2011). Over-expression of GGCT has previously been detected in a large-scale integration of cancer microarray data (Xu *et al.* 2007). Furthermore, a proteomic profiling of breast cancer demonstrated that GGCT was up-regulated and correlated with poor clinical outcome. Gromov *et al.* also suggested GGCT as a potential serological marker as GGCT is secreted into extracellular space (Gromov *et al.* 2010). Expression of GGCT has previously been detected only in epithelial tumors such as lung, esophagus, stomach, bile duct, and uterine cervix cancer (Amano *et al.* 2012).

Two isoforms of extracellular cyclophilin A (CypA) was detected to be significantly up-regulated in the oral cancer secretome. Expression of Multiple isoforms of CypA has been detected in the secretome of irradiated breast cancer cells and was shown to be associated with radiosensitivity of cancer cells (Chevalier *et al.* 2012). The CypA isoform identified in our analysis were similar in terms of pI and MW to those previously identified in breast cancer (Chevalier *et al.* 2012). Expression of CypA has been reported to augment cancer cell proliferation through interaction with the CD147

receptor which regulates a series of cell proliferation-related biological processes (Li *et al.* 2006). Furthermore, it is involved in the phosphorylation of ERK1/2 in a cell-dependant manner and stimulates NF- $\kappa$ B (Bahmed *et al.* 2012). CypA also renders cancer cells more resistant to hypoxia- and cisplatin- induced apoptosis (Choi *et al.* 2007). Over-expression of secreted CypA has been observed in pancreatic cancer, liver fluke-associated cholangiocarcinoma, neoplastic and HPV-immortalized oral keratinocytes (Rey *et al.* 1999; Li *et al.* 2006; Obchoei *et al.* 2011).

A ~17 kDa fragment of Acidic leucine-rich nuclear phosphoprotein 32 family member B (ANP32B) was found to be increased in the oral cancer secretome. The expression of this short ANP32B fragment has been reported to be associated with the stimulation of apoptosis (Shen *et al.*). ANP32B is characterized by its amino-terminal leucine-rich repeat domain and a carboxyl-terminal region that is enriched with acidic amino acid residues (Huyton and Wolberger 2007). It has been reported as a critical nuclear protein which is indispensable for cell cycle progression to S phase thus, implying that it is required for cancer cell proliferation (Sun *et al.* 2001). ANP32B has been shown to be a marker for poor prognostic outcome in breast cancer (Reilly *et al.*). This protein has also been associated with cellular apoptosis in a subcellular proteomic analysis of NSC606985 induced apoptotic acute myeloid leukemia cells (Yu *et al.* 2007). ANP32B has also been shown to be a novel substrate for caspase-3 (Shen *et al.* 2010). It has recently been shown that ANP32B inhibits ATRA-induced leukemic cell differentiation through blocking the retinoic acid receptor signaling (Yu *et al.* 2012).

Two isoforms of Protein SET were significantly increased in oral cancer secretome. Protein spot ID.15 was recognized as isoform 1 of Protein SET (unique sequence KKPRPPPALGPEETSASAGLPKK at position 14 - 35 was identified in

MS/MS, refer to Appendix A, spot ID. 15) but other isoform of Protein SET (spot ID. 12) was not determined. Protein SET is a potent antagonist of protein phosphatase 2A (PP2A) which is a critical tumor suppressor gene (Li *et al.* 1996). PP2A is implicated in diverse cellular processes including the modulation of the oncogenic signal transduction pathway (Schonthal 2001; Westermarck and Hahn 2008). As a result, inhibition of PP2A is believed to be associated with tumor formation. A recent study demonstrated that shRNA knock-down of Protein SET expression restored PP2A tumor suppressor activities culminating in cancer cell apoptosis by reducing Mcl-1 levels via dephosphorylation of Akt and cancer cells cytotoxicity. This study also reported that the peptide COG449 which mimics the antagonistic effects of Protein SET inhibited *in vivo* growth of Ramos cell non-Hodgkin lymphoma tumor xenografts in SCID mice (Christensen *et al.* 2011). Undoubtedly, Protein SET over-expression has deleterious effects on the survival of cancer patients and it has been reported in chronic lymphocytic leukemia, non-Hodgkin lymphoma, acute myeloid leukemia, and Wilm's tumor (Carlson *et al.* 1998; Christensen *et al.* 2011; Cristobal *et al.* 2012).

Low molecular weight heat shock protein 10 (Hsp10) was significantly increased in oral cancer secretome but up-regulation of its mRNA transcript level was not significant. Intracellular Hsp10 is invariably localized to the mitochondria and constitute the mitochondrial chaperonin complex which is implicated in mitochondrial protein folding. However, extracellular Hsp10 which is also known as early pregnant factor (EPF) is released from ovaries into the maternal serum within 24 hours after fertilization in several mammalian species (Cavanagh 1996; Morton 1998). Intriguingly, extracellular Hsp10/EPF exhibits a very distinct role against intracellular Hsp10 which is located in mitochondria. Extracellular Hsp10/EPF is able to modulate cancer cell proliferation and facilitate evasion of tumor immune surveillance by suppressing T-cell

activities (Corrao *et al.* 2010). Moreover, normal cellular Hsp10 is preferably localized in the matrix of mitochondria but Hsp10 reaches higher levels in the cytosol of tumor cells. This suggested that accumulation of Hsp10 in the cytosol of tumor cells is the incipient course for secretion into the extracellular space via a non-classical pathway (Jia *et al.* 2011). The extracellular Hsp10/EPF has been found in the serum of testicular cancer patients (Rolfe *et al.* 1983). Several findings also demonstrated that overexpression of Hsp10 in exocervix cancer, prostate cancer and large bowel cancer (Cappello *et al.* 2003a; Cappello *et al.* 2003b; Cappello *et al.* 2005).

Secreted follistatin like 1 (FSTL1) was detected to be increased significantly in the oral cancer secretome. The common role for follistatin or other follistatin like proteins is as an antagonist against activin activities. However, FSTL1 harbors only a single follistatin domain and has no antagonistic activity against activin (Kreidl *et al.* 2009). It is a novel proinflammatory molecule that induces the secretion of IL-1 $\beta$ , TNF- $\alpha$ , IL6 cytokines and also promotes revascularization in ischemic tissue (Miyamae *et al.* 2006; Ouchi *et al.* 2008). It has been reported to be up-regulated in gliomas and highly metastatic prostate cancer but acts as a potential tumor suppressor in ovarian and endometrial carcinomas (Trojan *et al.* 2005; Chan *et al.* 2009). In addition, a proteomic study on the secretome of HepG2 and Hep3B cells demonstrated that FSTL1 is the characteristic of mesenchymal Hep3B cells and may be a potential marker for EMT (Slany *et al.*).

Cofilin-1 (CFL1) and elongation factor 1-delta (EF-1-delta) were increased in the oral cancer secretome. CFL1 plays an important role in cytoskeletal organization by regulating F-actin depolymerization activities. It has been reported to be associated with cancer invasion, intravasation, and chemoresistance (Wang *et al.* 2006; Yan *et al.* 2007).

A multivariate analysis of 78 samples demonstrated that overexpression of CFL1 mRNA level was a significant prognosis marker for advanced epithelial ovarian cancer in individuals receiving therapy (Nishimura *et al.* 2011). In the context of the translation process, elongation factor 1-delta is an integral part of the elongation factor-1 protein complex that regulates the elongation step of protein synthesis by transferring aminoacyl-t RNA to the 80S ribosomes. It has been shown that the expression level of elongation factor-1 delta was significantly up-regulated in oesophageal carcinoma and associated with lymph node metastases, advanced disease stages and poorer prognosis (Ogawa *et al.* 2004). Thus, up-regulation of CFL1 and EF-1-delta may facilitate oral cancer tumorigenesis.

Guanine nucleotide binding protein subunit beta-2-like-1 (GNB2L1) which is also known as receptor for activated C kinase 1 (RACK1) was significantly increased in oral cancer secretome. This protein is crucial in cancer angiogenesis and anti-apoptosis (Berns *et al.* 2000). Furthermore, it has been reported to promote breast carcinoma migration via RhoA pathway and regulation of VEGF/Flt1-mediated cell migration via the PI3-K/Akt pathway (Cao *et al.* 2011; Wang *et al.* 2011). Its overexpression has also been detected in oral squamous cell carcinoma tissues (Wang *et al.* 2008).

A decrease of interleukin-1 receptor antagonist protein (IL-1RN) was observed in the oral cancer secretome with its mRNA transcript expression was also found to be decreased. IL-1RN inhibits the effect of interleukine-1 by competing for its corresponding receptor site. IL-1RN has been suggested as a novel therapeutic agent which functions by suppressing the effect of interleukin 1 (Lewis *et al.* 2006). Additionally, it has been reported as an antagonist of interleukin 6 which is implicated in the differentiation and growth of tumor cells (Jordan *et al.* 1995; Oka *et al.* 1996;

Reichner *et al.* 1996). Elaraj *et al.* reported that recombinant IL-1RN significantly inhibited the xenograft growth of interleukin-1 producing tumor in athymic nude mice (Elaraj *et al.* 2006). A study with 80 colorectal cancer patients and tissues demonstrated that serum IL-1ra may be a potent index to evaluate colorectal cancer progression (Ito and Miki 1999).

Non-glycosylated form of cystatin M (CST6), ~14 kDa was identified to be reduced significantly in the secretome of oral cancer. However, its mRNA expression was found to be up-regulated. It primarily functions as an endogenous inhibitor of lysosomal cysteine proteinases (Turk and Bode 1991). Additionally, it renders tumor cells more resistant to TNF- $\alpha$  induced apoptosis by inhibiting cathepsin B and also facilitates cancer invasion (Zhang *et al.* 2004; Vigneswaran *et al.* 2005). Down-regulation of cystatin M was first reported in breast cancer cells and proved to be a novel candidate tumor suppressor gene for breast cancer (Sotiropoulou *et al.* 1997; Zhang *et al.* 2004). Diminishing of cystatin M in invasive breast cancer cells was reported to be associated with the losses of the estrogen receptor, progesterone receptor, and HER4 (Ko *et al.* 2010). Followed by such unprecedented finding in breast cancer, significantly differential expression of cystatin M in oral carcinoma cell lines was also revealed with the use of oligonucleotide arrays (Zacharias *et al.* 2001). It was further demonstrated that there was up-regulation of cystatin M during progression from primary tumor to its advanced stage (Vigneswaran *et al.* 2003). A study on carcinogenic functions of cystatin M in oral cancer showed that siRNA knock-down expression of cystatin M in oral cancer cell lines promoted cell proliferation and *in vitro* invasion (Vigneswaran *et al.* 2006).

Most of the calcium  $\text{Ca}^{2+}$ -binding proteins, secreted protein acidic and rich in cysteine (SPARC), S100 calcium-binding protein A8 (S100-A8), Reticulocalbin-1 precursor (RCN-1), and calmodulin-like protein 3 (CALML3) were found to be decreased in the oral cancer secretome. Among them, SPARC and S100-A8 mRNA transcript showed similar change in expression. SPARC is a secreted glycoprotein which plays an important role in developing bones and teeth, cell differentiation, migration, and angiogenesis. It has been reported that SPARC inhibits the proliferation of normal and cancer cells as well as induces apoptosis of cancer cells (Yiu *et al.* 2001). Low concentrations of S100-A8 promotes cancer cell invasion and increases cell proliferation whereas high concentration of S100-A8 exerts proapoptotic effects on cancer cells (Ghavami *et al.* 2008). The exact function of RCN-1 remains unclear although it may play a role in normal cell behavior (Fukuda *et al.* 2007). Hirano *et al.* reported that lower expression of RCN-1 rendered non-small lung cancer cells more resistant to platinum cisplatin chemotherapy (Hirano *et al.* 2005). Calmodulin like proteins which are specifically expressed in epithelial cells show some similar functions as calmodulin. However, it has a ~8-fold lower affinity towards  $\text{Ca}^{2+}$  than calmodulin. It also functions as a specific light chain of human unconventional myosin X, which is implicated in cell signaling and cell motility (Rogers and Strehler 2001). Rogers *et al.* has suggested that human calmodulin like protein 3 (CALML3) is aberrantly regulated during terminal epithelial cell differentiation resulting in disrupted control of cell differentiation and cell immortalization (Rogers *et al.* 2001). Down-regulation of CALML3 was observed in cancer transformed human mammary epithelial cell lines induced by Kirsten sarcoma virus as well as in the majority of breast cancer cells (Yaswen *et al.* 1990; Rogers *et al.* 1999). Down-regulation of CALML3 in our data is further substantiated in a study that demonstrated a down-regulation of CALML3



expression in oral malignant transformation compared to expression of CALML3 in normal oral mucosa (Brooks *et al.* 2009).

## **CHAPTER 6**

## **CONCLUSION**

## 6.0 Conclusion

The secretome of three cancer cell lines (48T, 153T, H400) were profiled using 2D gel electrophoresis. By comparing 2D gel images of the secretome of cancer and its normal counterpart, thirty one proteins were observed to have changed in abundance in cancer cells. Proteins which are highly cancer associated and likely secreted were also validated in their transcript level. Among these proteins, differential expression of MMP-7, hnRNP-A2/B1 spliced variants, PRDX-1, LAMB3 subunit, TIMP-1, IL-1RN, S100-A8 and SPARC were found to be consistent and significant in both proteome and gene expression analysis. These proteins were known to be associated with cancer migration, immortalization, anti-apoptosis, and proliferation by many studies worldwide.

Early-detection of oral cancer via serological biomarkers is less invasive and more accessible. Therefore, discovery of oral cancer serological biomarkers is an ultimate goal for a number of researchers. In our study, MMP7, hnRNP-A2/B1 spliced variants, PRDX-1, LAMB3 subunit, TIMP-1, IL-1RN, S100-A8 and SPARC were detected to have significantly changed in the oral cancer cell lines secretome suggesting these proteins may also be secreted into the blood circulatory system. Thus, this work has demonstrated the significance of studying the cancer cell line secretome in identifying potential biomarker candidates.

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## **APPENDICES**

**SPOT ID. 1*****(MATRIX)  
(SCIENCES)* Mascot Search Results****Protein View**

Match to: **CADH2\_HUMAN** Score: **110** Expect: **1.6e-007**  
**Cadherin-2 precursor (Neural-cadherin) (N-cadherin) (CD325 antigen)**  
**(CDw325) - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **99747**; Calculated pI value: **4.64**  
 NCBI BLAST search of **CADH2\_HUMAN** against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **3%**

Matched peptides shown in **Bold Black**

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  1 MCRIAGALRT LLPLLAALLQ ASVEASGEIA LCKTGFPEDEV YSAVLSKDVBH
 51 EGQPLLNVKF SNCNGKRKVQ YSESEPADFK VDEEDGMVYAV RSFPLSSEHA
101 KFLIYAQDKE TQEKWQVAVK LSLKPTLTEE SVKESAEVEE IVFPRQFSKH
151 SGHLQRQKRD WVIPPINLPE NSRGPPFQEL VRIRSDRDKN LSLRYSVTGP
201 GADQPPTGIF IINPISGQLS VTKPLDREQI ARFHLRAHAV DINGNQVENP
251 IDIVINVIDM NDNRPEFLHQ VWNGTVPEGS KPGTYVMTVT AIDADDPNAL
301 NGMLRYRIVS QAPSTPSPNM FTINNETGDI ITVAAGLDRE KVQQYTLIIQ
351 ATDMEGNPTY GLSNTATAVI TVTDVNDNPP EFTAMTFYGE VPENRVDIIV
401 ANLTVTDKQD PHTPAWNAVY RISGGDPTGR FAIQTDPNNS DGLVTVVKPI
451 DFETNRMFVL TVAAENQVPL AKGIQHPPQS TATVSVTVID VNENPYFAPN
501 PKIIRQEEGL HAGTMLTFTT AQDPDRYMQQ NIRYTKLSDP ANWLKIDPVN
551 GQITTIAVLD RESPNVKNNI YNATFLASDN GIPPMMSGTGT LQIYLLDIND
601 NAPQVLPQEA ETCETPDPNS INITALDYDI DPNAGPFAFD LPLSPVTIKR
651 NWTITRLNGD FAQLNLKIKF LEAGIYEVPI IITDSGNPPK SNISILRVKV
701 CQCDSNGDCT DVDRIVGAGL GTGAI IAILL CIIILLILVL MFVVWMKRRD
751 KERQAKQLLI DPEDDVRDNI LKYDEEGGGE EDQDYDLSQL QQPDTVEPDA
801 IKPVGIRRM D ERPIHAEPQY PVRSAAPHPG DIGDFINEGL KAADNDPTAP
851 PYDSL LVFDY EGS GSTAGSL SSLNSSSSSGG EQDYDYLN DW GPRFKKLADM
901 YGGGDD

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Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
92 - 101	1102.51	1101.50	1101.55	-0.05	0	R.SFPLSSEHAK.F
102 - 114	1612.75	1611.74	1611.81	-0.07	1	K.FLIYAQDKETQEK.W
150 - 156	834.39	833.38	833.43	-0.04	0	K.HSGHLQR.Q

**SPOT ID. 2*****{MATRIX}***  
***{SCIENCE}*** Mascot Search Results**Protein View**Match to: **CATD\_HUMAN** Score: **492** Expect: **1e-045****Cathepsin D precursor (EC 3.4.23.5) [Contains: Cathepsin D light chain; Cathepsin D heavy chain] -**Nominal mass ( $M_r$ ): **44524**; Calculated pI value: **6.10**NCBI BLAST search of **CATD\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **47%**Matched peptides shown in **Bold Black**

**1** MQPSSLLPLA LCLLAAPASA LVR**IPLHKFT** **SIR**RTMSEVG GSVEDLIAKG  
**51** PVSK**YSQAVP** **AVTEGPIPEV** LKNYMDAQYY GEIGIGTPPQ CFTVVFDTGS  
**101** SNLWVPSIHC KLLDIACWIH HKYNSDKSST YVKNGTSEFDI HYGSGSLSGY  
**151** LSQDTVSVPC QSASSASALG GVKVER**QVFG** **EATKQPGITF** **IAAKFDGILG**  
**201** **MAYPRISVNN** **VLPVFDNLMQ** **QKLVDQNI**FS **FYLSRDPDAQ** **PGGELMLGGT**  
**251** **DSKYKGSLS** YLNVTRK**AYW** **QVHLDQVEVA** **SGLTLCKEGC** **EAIVDGTSL**  
**301** **MVGPVDEVRE** **LQKAIGAVPL** **IQGEYMIPCE** **KVSTLPAITL** **KLGGKGYKLS**  
**351** **PEDYTLKVSQ** AGKTLCLSGF MGMTDIPPPSG PLWILGDVFI **GRYYTVFDRD**  
**401** **NNRVGFAEAA** **RL**

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
24 - 33	1211.74	1210.73	1210.72	0.01	1	R.IPLHKFTSIR.R
55 - 72	1898.02	1897.01	1897.02	-0.01	0	K.YSQAVPAVTEGPIPEVLK.N
177 - 184	879.46	878.45	878.45	-0.00	0	R.QVFG <b>EATK.Q</b>
177 - 194	1906.04	1905.03	1905.04	-0.00	1	R.QVFG <b>EATKQPGITF</b> IAAK.F
185 - 194	1045.58	1044.58	1044.60	-0.02	0	K.QPGITFIAAK.F
195 - 205	1239.63	1238.62	1238.61	0.01	0	K.FDGILGMAYPR.I
195 - 205	1255.62	1254.61	1254.61	0.01	0	K.FDGILGMAYPR.I (M)
206 - 222	1975.03	1974.02	1974.02	-0.01	0	R.ISVNNVLPVFDNLM <b>QQK.L</b> (M)
223 - 235	1601.84	1600.83	1600.82	0.00	0	K.LVDQNI <b>FSFYLSR.D</b>
236 - 253	1787.81	1786.80	1786.80	-0.01	0	R.DPDAQ <b>PGGELMLGGT</b> DSK.Y
268 - 287	2317.11	2316.10	2316.16	-0.05	0	K.AYW <b>QVHLDQVEVA</b> SGLTLCK.E (C)
288 - 309	2334.09	2333.08	2333.09	-0.01	0	K.EGCEAI <b>VDGTSLM</b> VG <b>PVDEVR.E</b> (C)
288 - 309	2350.10	2349.09	2349.08	0.01	0	K.EGCEAI <b>VDGTSLM</b> VG <b>PVDEVR.E</b> (C); (M)
314 - 331	1989.01	1988.00	1988.01	-0.01	0	K.AIGAV <b>PLIQGEYMIPCEK.V</b> (C)
314 - 331	2005.01	2004.00	2004.01	-0.01	0	K.AIGAV <b>PLIQGEYMIPCEK.V</b> (C); (M)
349 - 357	1065.55	1064.54	1064.54	0.00	0	K.LSPED <b>YTLK.V</b>
393 - 399	963.46	962.46	962.45	0.01	0	R.Y <b>YTVFDR.D</b>
393 - 403	1462.68	1461.67	1461.66	0.01	1	R.Y <b>YTVFDRD</b> NNR.V
404 - 411	820.44	819.43	819.42	0.00	0	R.VG <b>FAEAAAR.L</b>
404 - 412	933.52	932.52	932.51	0.01	1	R.VG <b>FAEAAAR.L</b> .-

**SPOT ID. 3*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **TIMP2\_HUMAN** Score: **314** Expect: **6.4e-028****Metalloproteinase inhibitor 2 precursor (TIMP-2) (Tissue inhibitor of metalloproteinases 2) (CSC-21**Nominal mass ( $M_r$ ): **24383**; Calculated pI value: **7.45**NCBI BLAST search of TIMP2\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **32%**Matched peptides shown in **Bold Black**

**1** MGAAARTLRL ALGLLLLLATL LRPADACSCS PVHPQQAFCN ADVVIRAKAV  
**51** SE**KEVDSGND IYGNPIKRIQ** YEIKQIKMFK GPEKDIEFIY **TAPSSAVCGV**  
**101** **SLDVGGKKEY** LIAGKAEGDG KMHITLCDFI VPWDTLSTTQ **KKSLNHR****YQM**  
**151** **GCECK**ITRCP MIPCYISSPD ECLWMDWVTE KNINGHQAKF **FACIK****RSDG****S**  
**201** **CAWYRGAAPP** **KQEF**LDIEDP

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
54 - 68	1676.96	1675.95	1675.82	0.13	1	K.EVDSGNDIYGNPIKR.I
85 - 107	2328.21	2327.20	2327.14	0.07	0	K.DIEFIYTAPSSAVCGVSLDVGGK.K
148 - 155	1075.48	1074.47	1074.39	0.08	0	R.YQMGCECK.I 2 (C)
196 - 205	1257.63	1256.62	1256.54	0.09	1	K.RSDGSCAWYR.G (C)
197 - 205	1101.52	1100.51	1100.43	0.08	0	R.SDGSCAWYR.G (C)
206 - 220	1626.93	1625.92	1625.79	0.13	1	R.GAAPPKQEFLDIEDP.-

**SPOT ID. 4*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **FSTL1\_HUMAN** Score: **206** Expect: **4e-017****Follistatin-related protein 1 precursor (Follistatin-like 1) - Homo sapiens (Human)**Nominal mass ( $M_r$ ): **34963**; Calculated pI value: **5.39**NCBI BLAST search of FSTL1\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **12%**Matched peptides shown in **Bold Black**

**1** MWKRWLALAL ALVAVAVVRA EEELRSK**SKI CANVFCGAGR** ECAVTEKGEP  
**51** TCLCIEQCKP HKRPVCGSNG KTYLNHCELH RDACLTGSKI QVDYDGHCKE  
**101** KKSVPSPASP VVCYQSNRDE LRRRIIQWLE AEIIPDGWFS KGSNYSEILD  
**151** KYFKNFDNGD SRLDSSEFLK FVEQNETAIN ITTYPDQENN KLLRGLCVDA  
**201** LIELSDENAD WKLSFQEFK **CLNPSFNPPE KKCALEDETY** ADGAETEVDK  
**251** NRCVCACGNW VCTAMTCDGK NQKGAQTQTE EEMTR**YVQEL QKHQETAET**  
**301** KRVSTKEI

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
28 - 40	1439.64	1438.63	1438.68	-0.05	1	K.SKICANVFCGAGR.E 2 (C)
30 - 40	1224.52	1223.52	1223.55	-0.04	0	K.ICANVFCGAGR.E 2 (C)
221 - 232	1430.66	1429.65	1429.70	-0.05	1	K.CLNPSFNPPEKK.C (C)
286 - 299	1730.80	1729.79	1729.86	-0.07	1	R.YVQELQKHQETAET.T
293 - 299	842.48	841.47	841.39	0.08	0	K.HQETAET.T

**SPOT ID. 5*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **ROA2\_HUMAN** Score: **57** Expect: **0.029****Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1) - Homo sapiens (Human)**Nominal mass ( $M_r$ ): **37407**; Calculated pI value: **8.97**NCBI BLAST search of **ROA2\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **7%**Matched peptides shown in **Bold Black**

```

  1 MEKTLETVPL ERKKREKEQF RKLFIGGLSF ETTEESLRNY YEQWGKLTDC
 51 VVMRDPASKR SRGFGFVTFS SMAEVDAAAMA ARPHSIDGRV VEPKRAVARE
101 ESGKPGAHVT VKKLFVGGIK EDTEEHHLRD YFEEYGKIDT IEIITDRQSG
151 KKRGFQFVTF DDHDPVDKIV LQKYHTINGH NAEVRKALSR QEMQEVQSSR
201 SGRGGNFGFG DSRGGGGNFG PGPGSNFRGG SDGYGSGRGF GDGYNGYGGG
251 PGGGNFGGSP GYGGGRGGYG GGGPGYGNQG GGYGGGYDNY GGGNYGSGNY
301 NDFGNYNQQP SNYGPMKSGN FGGSRNMGPP YGGGNYGPPG SGGSGGYGGR
351 SRY

```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
23 - 38	1798.95	1797.95	1797.91	0.03	0	K.LFIGGLSFETTEESLR.N
204 - 213	1013.46	1012.45	1012.44	0.02	0	R.GGNFGFGDSR.G

**SPOT ID. 6*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **PRDX1\_HUMAN** Score: **187** Expect: **3.2e-015****Peroxiredoxin-1 (EC 1.11.1.15) (Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide reductase**Nominal mass ( $M_r$ ): **22096**; Calculated pI value: **8.27**NCBI BLAST search of PRDX1\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **23%**Matched peptides shown in **Bold Black**

**1** MSSGNAKIGH PAPNFK**ATAV MPDGQFK**DIS LSDYKGKYVV FFFYPLDFTF  
**51** VCPTEIIAFS DRAEEFKKLN **CQVIGASVDS** HFCHLAWVNT PKKQGGLGPM  
**101** NIPLVSDPKR TIAQDYGVLK **ADEGISFRGL** FIIDDKGILR **QITVNDLPVG**  
**151** RSVDETLRLV **QAFQFTDK**HG EVCPAGWKPG SDTIKPDVQK SKEYFSKQK

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
17 - 27	1164.56	1163.55	1163.56	-0.01	0	K.ATAVMPDGQFK.D
121 - 128	894.42	893.41	893.42	-0.01	0	K.ADEGISFR.G
141 - 151	1211.66	1210.65	1210.67	-0.02	0	R.QITVNDLPVGR.S
152 - 158	819.42	818.41	818.41	-0.00	0	R.SVDETLR.L
159 - 168	1196.62	1195.61	1195.62	-0.01	0	R.LVQAFQFTDK.H

**SPOT ID. 7*****(MATRIX)*  
*(SCIENCES)* Mascot Search Results****Protein View**

Match to: **CH10\_HUMAN** Score: **121** Expect: **1.3e-008**  
**10 kDa heat shock protein, mitochondrial (Hsp10) (10 kDa chaperonin) (CPN10)**  
**(Early-pregnancy facto**

Nominal mass ( $M_r$ ): **10925**; Calculated pI value: **8.89**  
 NCBI BLAST search of **CH10\_HUMAN** against nr  
**Unformatted sequence string for pasting into other applications**

**Taxonomy: Homo sapiens**

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **40%**

**Matched peptides shown in Bold Black**

**1** MAGQAFRK**FL PLEDRVLVER** SAAETVTK**GG IMLPEKSQ**GK VLQATVVAVG  
**51** SGSKGKGG**EI QPVSVKVGDK VLLPEYGGTK VVLDDKDYFL FRDGDILGKY**  
**101** VD

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
9 - 15	907.52	906.52	906.50	0.02	0	K.FLPLFDR.V
29 - 36	860.46	859.45	859.45	0.00	0	K.GGIMLPEK.S (M)
67 - 80	1475.79	1474.79	1474.80	-0.02	1	K.VGDKVLLPEYGGTK.V
71 - 80	1076.61	1075.61	1075.59	0.01	0	K.VLLPEYGGTK.V
81 - 92	1529.83	1528.82	1528.79	0.03	1	K.VVLDDKDYFLFR.D



**SPOT ID. 8*****{MATRIX}***  
***{SCIENCES}*** Mascot Search ResultsMatch to: **AN32B\_HUMAN** Score: **343** Expect: **8e-031****Acidic leucine-rich nuclear phosphoprotein 32 family member B (PHAPI2 protein)**  
**(Silver-stainable pr**Nominal mass ( $M_r$ ): **28770**; Calculated pI value: **3.94**NCBI BLAST search of **AN32B\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **23%**Matched peptides shown in **Bold Black**

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1 MDMKRRIHLE LRNRTPAAVR ELVLDNCKSN DGKIEGLTAE FVNLEFLSLI
51 NVGLISVSNL PKLPKLKKLE LSENIFGGL DMLAEKLPNL THLNLSGNKL
101 KDISTLEPLK KLECLKSLDL FNCEVTNLND YRESVFKLLP QLTYLDGYDR
151 EDQEAPDSDA EVDGVDEEEE DEEGEDEEDE DDEEGEEEF DEEDEDEDV
201 EGDEDDDEVS EEEEEFGLDE EDEDEDEDEE EEEGGKGEKR KRETDGED
251 D

```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
6 - 12	936.63	935.62	935.57	0.06	1	R.RIHLELR.N
76 - 86	1193.69	1192.69	1192.62	0.07	0	R.IFGGLDMLAEK.L
87 - 99	1420.88	1419.87	1419.78	0.09	0	K.LPNLTHLNLSG <b>NKL</b> .L
117 - 132	1973.03	1972.02	1971.90	0.12	0	K.SLDL <b>FNCEVTNLNDYR</b> .E (C)
138 - 150	1566.91	1565.90	1565.81	0.09	0	K.LLPQ <b>LT</b> YLDGYDR.E

**SPOT ID. 9*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **ROA2\_HUMAN** Score: **92** Expect: **1e-005****Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1) - Homo sapiens (Human)**Nominal mass ( $M_r$ ): **37407**; Calculated pI value: **8.97**NCBI BLAST search of **ROA2\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **15%**Matched peptides shown in **Bold Black**

```

1 MEKTLETVPL ERKKREKEQF RKLFIGGLSF ETTEESLRNY YEQWGKLTDC
51 VVMRDPASKR SRGFGFVTF S MAEVDAAAMA ARPHSIDGRV VEPKRAVARE
101 ESGKPGAHVT VKKLFVGGIK EDTEEHHLRD YFEEYGKIDT IEIITDRQSG
151 KKRGFVTF DDHDPVDKIV LQKYHTINGH NAEVRKALSR QEMQEVQSSR
201 SGRGGNFGFG DSRGGGNFG PGPGSNFRGG SDGYGSGRGF GDGYNGYGGG
251 PGGGNFGGSP GYGGGRGGYG GGGPGYGNQG GGYGGGYDNY GGGNYGSGNY
301 NDFGNYNQQP SNYGPMKSGN FGSRNMGGP YGGGNYGPGG SGGSGGYGGR
351 SRY

```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
4 - 12	1057.52	1056.52	1056.58	-0.06	0	K.TLETVPLER.K
16 - 21	836.39	835.38	835.42	-0.04	1	R.EKEQFR.K
100 - 112	1338.65	1337.65	1337.69	-0.05	0	R.EESGKPGAHVTVK.K
100 - 113	1466.74	1465.74	1465.79	-0.05	1	R.EESGKPGAHVTVKK.L
138 - 147	1188.60	1187.59	1187.64	-0.05	0	K.IDTIEIITDR.Q
187 - 200	1648.75	1647.75	1647.80	-0.05	1	K.ALSRQEMQEVQSSR.S
191 - 200	1221.51	1220.50	1220.55	-0.04	0	R.QEMQEVQSSR.S

**SPOT ID. 10*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**

Match to: **PCNA\_HUMAN** Score: **193** Expect: **1.3e-014**  
**Proliferating cell nuclear antigen (PCNA) (Cyclin) - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **28750**; Calculated pI value: **4.57**  
 NCBI BLAST search of **PCNA\_HUMAN** against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **20%**

Matched peptides shown in **Bold Black**

1 MFEARLVQGS ILKKVLEALK DLINEACWDI SSSGVNLQSM DSSHVSLVQL  
 51 **TLRSEGFDTY** RCDRNLAMGV NLTSMSKILK **CAGNEDIITL** **RAEDNADTLA**  
 101 **LVFEAPNQEK** VSDYEMKLMD LDVEQLGIPE QEYSCVVK**MP** **SGEFAR**ICRD  
 151 LSHIGDAVVI SCAKDGVKFS ASGELGNGNI KLSQTSNVDK EEEAVTIEMN  
 201 EPVQLTFALR **YLNFFTK**ATP LSSTVTLSMS ADVPLVVEYK IADMGHLKYY  
 251 LAPKIEDEEG S

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
54 - 61	974.47	973.46	973.41	0.05	0	R.SEGFDTYR.C
81 - 91	1261.69	1260.68	1260.61	0.07	0	K.CAGNEDIITLR.A (C)
92 - 110	2075.10	2074.09	2073.99	0.10	0	R.AEDNADTLALVFEAPNQEK.V
139 - 146	910.45	909.44	909.40	0.04	0	K.MPSGEFAR.I (M)
211 - 217	932.54	931.53	931.48	0.05	0	R.YLNFFTK.A

**SPOT ID. 11*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **MMP7\_HUMAN** Score: **359** Expect: **2e-032****Matrilysin precursor (EC 3.4.24.23) (Pump-1 protease) (Uterine metalloproteinase) (Matrix metallopr**Nominal mass (M<sub>r</sub>): **29658**; Calculated pI value: **7.74**NCBI BLAST search of MMP7\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **29%**Matched peptides shown in **Bold Black**

**1** MRLTVLCAVC LLPGSLALPL PQEAGGMSEL QWEQAQDYLK **RFYLYDSETK**  
**51** NANSLEAKLK EMQK**FFGLPI** **TGMLNSRVIE** **IMQKPRCGVP** **DVAEYSLFPN**  
**101** **SPKWTSKVVT** YRIVSYTRDL **PHITVDR**LVS KALNMWGKEI **PLHFRKVVWG**  
**151** **TADIMIGFAR** GAHGDSYPFD GPGNTLAHAF APGTGLGGDA HFDEDERWTD  
**201** GSSLGINFLY AATHELGHSL GMGHSSDPNA VMYPTYGNGD PQNFKLSQDD  
**251** IKGIQKLYGK RSNSRKK

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
41 - 50	1321.66	1320.65	1320.63	0.01	1	K.RFYLYDSETK.N
42 - 50	1165.56	1164.56	1164.53	0.02	0	R.FYLYDSETK.N
65 - 77	1452.79	1451.79	1451.76	0.03	0	K.FFGLPITGMLNSR.V
65 - 77	1468.79	1467.78	1467.75	0.03	0	K.FFGLPITGMLNSR.V (M)
78 - 86	1113.67	1112.66	1112.64	0.02	0	R.VIEIMQKPR.C
78 - 86	1129.66	1128.65	1128.63	0.02	0	R.VIEIMQKPR.C (M)
87 - 103	1879.92	1878.91	1878.88	0.03	0	R.CGVPDVAEYSLFPNSPK.W (C)
119 - 127	1065.59	1064.58	1064.56	0.02	0	R.DLPHITVDR.L
139 - 145	911.53	910.52	910.50	0.02	0	K.EIPLHFR.K
147 - 160	1535.83	1534.82	1534.80	0.02	0	K.VVWGTADIMIGFAR.G
147 - 160	1551.82	1550.81	1550.79	0.02	0	K.VVWGTADIMIGFAR.G (M)

**SPOT ID. 12*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **SET\_HUMAN** Score: **378** Expect: **2.5e-034****Protein SET (Phosphatase 2A inhibitor I2PP2A) (I-2PP2A) (Template-activating factor I) (TAF-I) (HLA**Nominal mass ( $M_r$ ): **33469**; Calculated pI value: **4.23**NCBI BLAST search of **SET\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **20%**Matched peptides shown in **Bold Black**

**1** MAPKQOSPLP PQKKKPRPPP ALGPEETSAS AGLPKKGEKE QQEAIEHIDE  
**51** VQNEIDRLNE QASEEILKVE QKYNK**LRQPF** **FQKRSELI**AK IPNFWVTTFV  
**101** NHPQVSALLG EEDEEALHYL TR**VEVTEFED** **IKSGYRIDFY** **FDENPYFENK**  
**151** **VLSKEFHLNE** **SGDPSSK**STE IKWKSGKDLT KRSSQTQNK SRKRQHEEPE  
**201** SFFTWFSTDHS DAGADELGEV IKDDIWPNPL QYYLVPDMD EEEGEEEDDD  
**251** DDEEEEGLED IDEEGDEDEG EEDEDDDEGE EGEEDEGEDD

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
76 - 83	1063.58	1062.57	1062.60	-0.03	1	K.LRQFFFQK.R
78 - 84	950.49	949.48	949.51	-0.03	1	R.QFFFQKR.S
84 - 90	816.47	815.46	815.49	-0.02	1	K.RSELIK.I
123 - 132	1208.57	1207.56	1207.60	-0.04	0	R.VEVTEFEDIK.S
123 - 136	1671.77	1670.77	1670.82	-0.05	1	R.VEVTEFEDIKSGYR.I
137 - 150	1840.76	1839.75	1839.80	-0.05	0	R.IDFYFDENPYFENK.V
151 - 167	1873.87	1872.86	1872.92	-0.06	1	K.VLSKEFHLNESGDPSSK.S
155 - 167	1446.60	1445.60	1445.64	-0.05	0	K.EFHLNESGDPSSK.S

**SPOT ID. 13*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **GBLP\_HUMAN** Score: **265** Expect: **5.1e-023****Guanine nucleotide-binding protein subunit beta 2-like 1 (Guanine nucleotide-binding protein subuni**Nominal mass ( $M_r$ ): **35055**; Calculated pI value: **7.60**NCBI BLAST search of **GBLP\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **23%**Matched peptides shown in **Bold Black**

**1** MTEQMTLRGT LKGHNWVWVQ IATTPQFPDM ILSASRD**KTI** **IMWKLTRDET**  
**51** **NYGIPQR**ALR GHSHFVSDVV ISSDGQFALS GSWDGTLLRW DLTTGTTTTRR  
**101** FVGH**TKDVLS** **VAFSSDNR**QI VSGSRDKTIK **LWNTLGVCKY** **TVQDESHSEW**  
**151** **VSCVR**FSPNS SNPIIVSCGW DKLVKVVWVLA NCKLKTNHIG HTGYLNTVTV  
**201** SPDGSLCASG GKDGQAMLWD LNEGKHLYTL DGGDIINALC FSPNR**YWLCA**  
**251** **ATGPSIK**IWD LEGKIIVDEL KQEVISTSSK AEPPQCTSLA WSADGQTLFA  
**301** GYTDNLVR**VW** **QVTIGTR**

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
39 - 44	807.40	806.39	806.44	-0.04	0	K.TIIMWK.L (M)
48 - 57	1192.57	1191.57	1191.55	0.01	0	R.DETNYGIPQR.A
107 - 118	1309.66	1308.65	1308.63	0.02	0	K.DVLSVAFSSDNR.Q
131 - 139	1033.52	1032.52	1032.54	-0.03	0	K.LWNTLGVCK.Y
140 - 155	1981.89	1980.89	1980.86	0.02	0	K.YTVQDESHSEWVSCVR.F (C)
246 - 257	1309.66	1308.65	1308.65	-0.00	0	R.YWLCAATGPSIK.I
246 - 257	1366.69	1365.68	1365.68	0.01	0	R.YWLCAATGPSIK.I (C)
309 - 317	1059.61	1058.60	1058.59	0.02	0	R.VWQVTIGTR.-

**SPOT ID. 14*****{MATRIX}*  
*{SCIENCES}* Mascot Search Results****Protein View**

Match to: **PCNA\_HUMAN** Score: **125** Expect: **5.1e-009**  
**Proliferating cell nuclear antigen (PCNA) (Cyclin) - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **28750**; Calculated pI value: **4.57**  
 NCBI BLAST search of **PCNA\_HUMAN** against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **13%**

Matched peptides shown in **Bold Black**

```

1 MFEARLVQGS ILKKVLEALK DLINEACWDI SSSGVNLQSM DSSHVSLVQL
51 TLRSEGFDTY RCDRNLMAGV NLTSMKILK CAGNEDIITL RAEDNADTLA
101 LVFEAPNQEK VSDYEMKLM DVEQLGIPE QEYSCVVKMP SGEFARICRD
151 LSHIGDAVVI SCAKDGVKFS ASGELGNGNI KLSQTSNVDK EEEAVTIEMN
201 EPVQLTFALR YLNFFTKATP LSSTVTLSMS ADVPLVVEYK IADMGHLKYY
251 LAPKIEDEEG S

```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
54 - 61	974.51	973.50	973.41	0.08	0	R.SEGFDTYR.C
92 - 110	2075.18	2074.18	2073.99	0.19	0	R.AEDNADTLALVFEAPNQEK.V
211 - 217	932.56	931.56	931.48	0.08	0	R.YLNFFTK.A

**SPOT ID. 15*****(MATRIX)*  
*(SCIENCES)* Mascot Search Results****Protein View**Match to: **SET\_HUMAN** Score: **239** Expect: **2e-020****Protein SET (Phosphatase 2A inhibitor I2PP2A) (I-2PP2A) (Template-activating factor I) (TAF-I) (HLA**Nominal mass (M<sub>r</sub>): **33469**; Calculated pI value: **4.23**NCBI BLAST search of **SET\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **27%**Matched peptides shown in **Bold Black**

**1** MAPKQOSPLP PQQ**KKPRPPP** **ALGPEETSAS** **AGLPKK**GEKE QQEAIIEHIDE  
**51** VQNEIDRLNE QASEEILKVE QKYN**LRQPF** **FQKR**SELI AK IPNFWVTTFV  
**101** NHPQVSALLG EEDEEALHYL TR**VEVTEFED** **IKSGYRIDFY** **FDENPYFENK**  
**151** VLSK**EFHLNE** **SGDPSSK**STE IKWKSGKDLT KR**SSQTQNK**A **SRKRQ**HEEPE  
**201** SFFTWF~~TD~~H~~S~~ DAGADELGEV IKDDIWP~~N~~PL QYYLVPD~~M~~DD EE~~G~~E~~G~~E~~E~~EDDD  
**251** DDEEEEGLED IDEEGDEDEG EEDEDDDEGE EGE~~E~~DEGEDD

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
14 - 35	2228.26	2227.26	2227.23	0.02	1	K.KKPRPPPALGPEETSASAGLPK.K
15 - 35	2100.17	2099.16	2099.14	0.02	0	K.KPRPPPALGPEETSASAGLPK.K
15 - 36	2228.26	2227.26	2227.23	0.02	1	K.KPRPPPALGPEETSASAGLPKK.G
76 - 83	1063.62	1062.61	1062.60	0.01	1	K.LRQPFQK.R
78 - 84	950.53	949.52	949.51	0.01	1	R.QPFFQK.R.S
123 - 132	1208.62	1207.62	1207.60	0.02	0	R.VEVTEFEDIK.S
137 - 150	1840.83	1839.83	1839.80	0.03	0	R.IDFYFDENPYFENK.V
155 - 167	1446.66	1445.65	1445.64	0.01	0	K.EFHLNESGDPSSK.S
183 - 192	1106.56	1105.56	1105.55	0.01	1	R.SSQTQNKASR.K



**SPOT ID. 16*****{MATRIX}*  
*{SCIENCES}* Mascot Search Results****Protein View**

Match to: **UBIQ\_HUMAN** Score: **211** Expect: **1.3e-017**  
**Ubiquitin - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **8560**; Calculated pI value: **6.56**  
 NCBI BLAST search of UBIQ\_HUMAN against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **50%**

Matched peptides shown in **Bold Black**

**1** MQIFVKTLTG **K**TITLEVEPS DTIENVKAKI **Q**DKEGIPPQ **Q**RLIFAGKQL  
**51** EDGRTLSDYN IQ**K**ESTLHLV **L**R**L**RGG

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
12 - 27	1787.86	1786.85	1786.92	-0.07	0	K.TITLEVEPSDTIENVK.A
30 - 42	1523.72	1522.71	1522.77	-0.06	1	K.IQDKEGIPPQQR.L
34 - 42	1039.49	1038.49	1038.51	-0.02	0	K.EGIPPQQR.L
64 - 72	1067.58	1066.57	1066.61	-0.04	0	K.ESTLHLVLR.L

SPOT ID. 17*{MATRIX}*  
*{SCIENCES}* Mascot Search Results**Protein View**Match to: **SSB\_HUMAN** Score: **279** Expect: **2e-024****Single-stranded DNA-binding protein, mitochondrial precursor (Mt-SSB) (MtSSB) (PWP1-interacting pro**Nominal mass ( $M_r$ ): **17249**; Calculated pI value: **9.59**NCBI BLAST search of SSB\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **43%**Matched peptides shown in **Bold Black**

**1** MFRRPVLQVL RQFVRHESET TTSLVLESL NRVHLLGRVG **QDPVLRQVEG**  
**51** **KNPVTIFSLA TNEMWRSGDS EVYQLGDVSQ KTTWHRISVF RPGLRDVAYQ**  
**101** **YVKKGSRIYL EGKIDYGEYM DKNNVRRQAT TIIADNIIFL SDQTKEKE**

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
39 - 46	883.55	882.54	882.49	0.05	0	R.VGQDPVLR.Q
52 - 66	1794.92	1793.91	1793.88	0.03	0	K.NPVTIFSLATNEMWR.S (M)
67 - 81	1611.84	1610.83	1610.74	0.09	0	R.SGDSEVYQLGDVSQK.T
87 - 95	1044.68	1043.68	1043.62	0.05	0	R.ISVFRPGLR.D
96 - 103	985.55	984.54	984.49	0.05	0	R.DVAYQYVK.K
114 - 122	1133.54	1132.53	1132.47	0.06	0	K.IDYGEYMDK.N

**SPOT ID. 18*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **HDGF\_HUMAN** Score: **159** Expect: **2e-012****Hepatoma-derived growth factor (HDGF) (High-mobility group protein 1-like 2) (HMG-1L2) - Homo sapie**Nominal mass ( $M_r$ ): **26772**; Calculated pI value: **4.70**NCBI BLAST search of HDGF\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **27%**Matched peptides shown in **Bold Black**

**1** MSRSNRQKEY **KCGDLVFAKM** **KGYPHWPARI** DEMPEAAVKS TANKYQVFFF  
**51** GTHETAFLGP **KDLFPYEESK** **EKFGKPNKRK** **GFSEGLWEIE** **NNPTVKASGY**  
**101** QSSQKKSCVE EPEPEPEAAE GDGDKKGNAE GSSDEEGKLV IDEPAKENE  
**151** KGALKRRAGD LLEDSPKRPK EAENPEGEEK **EAATLEVERP** **LPMEVEKNST**  
**201** PSEPGSGRGP PQEEEEEEDE EEEATKEDAE APGIRDHESL

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
12 - 21	1127.59	1126.58	1126.55	0.03	1	K.CGDLVFAKMK.G (M)
22 - 29	983.54	982.53	982.48	0.05	0	K.GYPHWPARI
62 - 70	1127.59	1126.58	1126.52	0.06	0	K.DLFPYEESK.E
73 - 79	846.52	845.51	845.49	0.02	1	K.FGKPNKR.K
81 - 96	1819.99	1818.98	1818.88	0.10	0	K.GFSEGLWEIENNPTVK.A
181 - 197	1941.12	1940.11	1939.99	0.12	0	K.EAATLEVERPLPMEVEK.N

SPOT ID. 19*(MATRIX)  
(SCIENCES)* Mascot Search Results**Protein View**Match to: **PPIA HUMAN** Score: **439** Expect: **2e-040****Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (Rotamase A) (Cyclophilin A) (Cyclosp**Nominal mass (M<sub>r</sub>): **18001**; Calculated pI value: **7.68**NCBI BLAST search of **PPIA HUMAN** against nrUnformatted [sequence string](#) for pasting into other applicationsTaxonomy: [Homo sapiens](#)

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **39%**Matched peptides shown in **Bold Black**

```
1  MVNPTVFFDI AVDGEPLGRV SFELFADKVP KTAENFRALS TGEKGFQYKG
51 SCFHRIIPGF MCQGGDFTRH NGTGGKSIYG EKFEDEFIL KHTGPGILSM
101 ANAGPNTNGS QFFICTAKTE WLDGKHVVFG KVKEGMNIVE AMERFQSRNG
151 KTSKKITAD CGQLE
```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
20 - 28	1055.59	1054.58	1054.53	0.05	0	R.VSFELFADK.V
20 - 31	1379.82	1378.81	1378.75	0.06	1	R.VSFELFADKVPK.T
56 - 69	1598.83	1597.82	1597.74	0.08	0	R.IIPGFMCQGGDFTR.H (C)
56 - 69	1614.82	1613.82	1613.73	0.08	0	R.IIPGFMCQGGDFTR.H (C); (M)
77 - 91	1832.00	1831.00	1830.90	0.09	1	K.SIYGKFEDEFILK.H
83 - 91	1154.63	1153.62	1153.57	0.05	0	K.FEDEFILK.H
132 - 144	1505.82	1504.81	1504.74	0.08	1	K.VKEGMNIVEAMER.F
132 - 144	1521.82	1520.81	1520.73	0.08	1	K.VKEGMNIVEAMER.F (M)
134 - 144	1278.65	1277.64	1277.57	0.06	0	K.EGMNIVEAMER.F
134 - 144	1294.64	1293.63	1293.57	0.06	0	K.EGMNIVEAMER.F (M)
155 - 165	1247.69	1246.68	1246.62	0.06	1	K.KITADCGQLE.- (C)
156 - 165	1119.57	1118.56	1118.53	0.03	0	K.ITADCGQLE.- (C)

**SPOT ID. 20*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **COF1\_HUMAN** Score: **73** Expect: **0.0009****Cofilin-1 (Cofilin, non-muscle isoform) (18 kDa phosphoprotein) (p18) - Homo sapiens (Human)**Nominal mass ( $M_r$ ): **18491**; Calculated pI value: **8.22**NCBI BLAST search of COF1\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **6%**Matched peptides shown in **Bold Black**

**1** MASGVAVSDG VIKVFNDMKV RKSSTPEEVK KRKKAVLFCL SEDKKNIIIE  
**51** EGKEILVGDV GQTVDDPYAT FVKMLPKDC **RYALYDATYE** **TKESKKEDLV**  
**101** FIFWAPESAP LKSKMIYASS KDAIKKKLTG IKHELQANCY EEVKDRCTLA  
**151** EKLGGSAVIS LEGKPL

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
82 - 92	1337.69	1336.69	1336.62	0.07	0	R.YALYDATYETK.E

SPOT ID. 21*(MATRIX)*  
*(SCIENCES)* Mascot Search Results**Protein View**Match to: **EF1D\_HUMAN** Score: **126** Expect: **4e-009****Elongation factor 1-delta (EF-1-delta) (Antigen NY-CO-4) - Homo sapiens (Human)**Nominal mass ( $M_r$ ): **31103**; Calculated pI value: **4.90**NCBI BLAST search of EF1D\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **28%**Matched peptides shown in **Bold Black**

**1** MATNFLAHEK IWFDKFK**YDD** **AERRFYEQMN** **GPVAGASRQE** NGASVILRDI  
**51** ARARENIQ**KS** **LAGSSGPGAS** **SGTSGDHGEL** **VVRIASLEVE** **NQSLR**GVVQE  
**101** LQQAISKLEA RLNVLEKSSP GHR**ATAPQTQ** **HVSPMRQVEP** PAKKPATPAE  
**151** DDEDDIDLF GSDNEEDKE AAQLREERLR QYAEKKAKKP ALVAKSSILL  
**201** DVKPWDETD MAQLEACVRS IQLDGLVWGA SK**LVPVGYGI** **RKLQIQCVVE**  
**251** DDKVGTDLLE EEITKFEHV QSVDIAAFNK I

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
18 - 24	924.41	923.40	923.41	-0.01	1	K.YDDAERR.F
25 - 38	1526.70	1525.69	1525.70	-0.01	0	R.FYEQMNGPVAGASR.Q
60 - 83	2185.05	2184.04	2184.04	-0.00	0	K.SLAGSSGPGASSGTSGDHGELVVR.I
84 - 95	1358.72	1357.72	1357.72	-0.00	0	R.IASLEVENQSLR.G
124 - 136	1423.71	1422.70	1422.70	-0.00	0	R.ATAPQTQHVSPMR.Q
233 - 241	973.58	972.57	972.58	-0.01	0	K.LVPVGYGIR.K

SPOT ID. 22*{MATRIX}*  
*{SCIENCES}* Mascot Search Results**Protein View**

Match to: **CG024\_HUMAN** Score: **156** Expect: **4e-012**  
**Uncharacterized protein C7orf24 - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **20994**; Calculated pI value: **5.07**  
 NCBI BLAST search of CG024\_HUMAN against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **25%**

Matched peptides shown in **Bold Black**

1 MANS**GCKDVT** GPDEESFLYF AYGSNLLTER IHLR**NPSAAF** **FCVARLQDFK**  
 51 **LDFGNSQGK**T SQTWHGGIAT IFQSPGDEVW GVVWKMNKS**N** LNSLDEQEGV  
 101 KSGMYVVIEV KVATQEGKEI TCRSYLMTNY ESAPPSPQYK KIICMGAK**EN**  
 151 **GLPLEYQEK**L KAIEPNDYTG **KVSEEIEDII** **KKGETQTL**

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
35 - 45	1239.61	1238.61	1238.59	0.02	0	R.NPSAAFFCVAR.L (C)
46 - 59	1596.82	1595.82	1595.79	0.02	1	R.LQDFKLDFGNSQK.T
149 - 159	1319.66	1318.66	1318.64	0.02	0	K.ENGLPLEYQEK.L
172 - 182	1302.65	1301.64	1301.71	-0.07	1	K.VSEEIEDIIKK.G

SPOT ID. 23*(MATRIX)*  
*(SCIENCES)* Mascot Search Results**Protein View**Match to: PPIA\_HUMAN Score: **116** Expect: **4e-008****Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (Rotamase A) (Cyclophilin A) (Cyclosp**Nominal mass ( $M_r$ ): **18001**; Calculated pI value: **7.68**NCBI BLAST search of PPIA\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **27%**Matched peptides shown in **Bold Black**

**1** MVNPTVFFDI AVDGEPLGRV SFELFADKVP KTAENFRALS TGEKGFYKKG  
**51** SCFHRIIPGF **MCQGGDFTR**H NGTGGKSIYG EK**FEDENFIL** KHTGPGILSM  
**101** ANAGPNTNGS QFFICTAKTE WLDGKHVVFG KVK**EGMNIVE** **AMER**FGSRNG  
**151** KTSK**KITIAD** **CGQLE**

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
56 - 69	1598.84	1597.83	1597.74	0.09	0	R.IIPGFMCQGGDFTR.H (C)
56 - 69	1614.83	1613.82	1613.73	0.09	0	R.IIPGFMCQGGDFTR.H (C); (M)
83 - 91	1154.63	1153.63	1153.57	0.06	0	K.FEDENFILK.H
134 - 144	1278.68	1277.68	1277.57	0.10	0	K.EGMNIVEAMER.F
155 - 165	1247.71	1246.71	1246.62	0.08	1	K.KITIADCGQLE.- (C)



**SPOT ID. 24*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **GSTO1\_HUMAN** Score: **64** Expect: **0.006****Glutathione transferase omega-1 (EC 2.5.1.18) (GSTO 1-1) - Homo sapiens (Human)**Nominal mass ( $M_r$ ): **27548**; Calculated pI value: **6.23**NCBI BLAST search of GSTO1\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **12%**Matched peptides shown in **Bold Black**

**1** MSGESARSLG **KGSAPPGPVP EGSIRI**YSMR **FCPFAER**TRL VLKAKGIRHE  
**51** VININLKNKP EWFFKKNPFG LVPVLENSQG QLIYESAITC EYLDEAYPGK  
**101** KLLPDDPYEK ACQKMILELF **SKVPSLVGSF IRSQNKEDYA** GLKEEFRKEF  
**151** TKLEEVLTNK KTTFFGGNSI SMIDYLIWPW FERLEAMKLN ECVDHTPKLK  
**201** LWMAAMKEDP TVSALLTSEK DWQGFLELYL QNSPEACDYG L

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
12 - 25	1320.69	1319.68	1319.68	-0.00	0	K.GSAPPGPVPEGSIR.I
31 - 37	869.38	868.38	868.39	-0.01	0	R.FCPFAER.T
31 - 37	926.41	925.41	925.41	-0.01	0	R.FCPFAER.T (C)
123 - 132	1074.63	1073.62	1073.62	-0.00	0	K.VPSLVGSFIR. <b>S</b>

SPOT ID. 25*{MATRIX}*  
*{SCIENCES}* Mascot Search Results**Protein View**

Match to: **CYTM\_HUMAN** Score: **264** Expect: **6.4e-023**  
**Cystatin-M precursor (Cystatin-6) (Cystatin-E) - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **16500**; Calculated pI value: **8.31**  
 NCBI BLAST search of CYTM\_HUMAN against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **51%**

Matched peptides shown in **Bold Black**

1 MARNLPLAL GLALVAFCLL ALPRDARARP QERMV**GELRD LSPDDPQVQK**  
 51 AAQAAVASYN MGSNSIYYFR DTHI**IKAQSQ LVAGIKYFLT MEMGSTDCRK**  
 101 TRVTGDHVDL TTCPLAAG**AQ QEKLR**CDFEV LVVPWQNSSQ LLKHNCVQM

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
34 - 50	1926.87	1925.86	1925.95	-0.09	1	R.MV <b>GELRD</b> LSPDDPQVQK.A
34 - 50	1942.86	1941.85	1941.95	-0.09	1	R.MV <b>GELRD</b> LSPDDPQVQK.A (M)
51 - 70	2183.93	2182.92	2183.01	-0.09	0	K.AAQAAVASYNMGSNSIYYFR.D
87 - 99	1610.60	1609.59	1609.66	-0.07	0	K.YFL <b>TMEMGSTDCR</b> .K (C)
87 - 100	1738.68	1737.67	1737.75	-0.08	1	K.YFL <b>TMEMGSTDCR</b> K.T (C)
101 - 123	2468.10	2467.10	2467.21	-0.12	1	K.TRVTGDHVDL <b>TTCPLAAGAAQ</b> QEK.L (C)
103 - 123	2210.99	2209.98	2210.06	-0.08	0	R.VTGDHVDL <b>TTCPLAAGAAQ</b> QEK.L (C)
103 - 125	2480.13	2479.12	2479.25	-0.13	1	R.VTGDHVDL <b>TTCPLAAGAAQ</b> QEKLR.C (C)

**SPOT ID. 26*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**Match to: **ILEU\_HUMAN** Score: **475** Expect: **5.1e-044****Leukocyte elastase inhibitor (LEI) (Serpin B1) (Monocyte/neutrophil elastase inhibitor) (M/NEI) (EI)**Nominal mass ( $M_r$ ): **42715**; Calculated pI value: **5.90**NCBI BLAST search of **ILEU\_HUMAN** against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **27%**Matched peptides shown in **Bold Black**

**1** MEQLSSANTR FALDLFLALS ENNPAGNIFI SPFSISSAMA MVFLGTRGNT  
**51** AAQLSK**TFHF** **NTVEEVHSRF** **QSLNADINKR** GASYILKLAN RLYGEK**TYNF**  
**101** **LPEFLVSTQK** **TYGADLASVD** **FQHASEDARK** TINQWVKGQT EGKIPELLAS  
**151** GMVDNMTKLV LVNAIYFKGN WKDKFM**KEAT** **TNAPFR**LNKK DRKTVKMMYQ  
**201** KKK**FAYGYIE** **DLKCRVLELP** YQGEELSMVI LLPDDIEDES TGLKKIEEQL  
**251** TLEKLHEWTK PENLDFIEVN VSLPR**FKLEE** **SYTLNSDLAR** LGVQDLFNSS  
**301** KADLSGMSG A RDIFISKIVH KSFVEVNEEG TEAAAATAGI ATFCMLMPEE  
**351** NFTADHPFLF FIR**HNSSGSI** **LFLGR**FSSP

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
57 - 69	1602.77	1601.77	1601.76	0.01	0	K.TFHFNTVEEVHSR.F
70 - 80	1305.69	1304.69	1304.68	0.00	1	R.FQSLNADINKR.G
97 - 110	1686.87	1685.86	1685.87	-0.00	0	K.TYNFLPEFLVSTQK.T
111 - 129	2052.93	2051.92	2051.92	0.00	0	K.TYGADLASVDFQHASEDAR.K
111 - 130	2181.03	2180.02	2180.01	0.01	1	K.TYGADLASVDFQHASEDARK.T
178 - 186	1006.49	1005.48	1005.49	-0.00	0	K.EATTNAPFR.L
204 - 213	1218.61	1217.60	1217.60	0.00	0	K.FAYGYIEDLK.C
276 - 290	1785.90	1784.90	1784.89	0.00	1	R.FKLEESYTLNSDLAR.L
364 - 375	1287.68	1286.67	1286.67	-0.00	0	R.HNSSGSILFLGR.F

SPOT ID. 27*{MATRIX}*  
*{SCIENCES}* Mascot Search Results**Protein View**

Match to: **CALL3\_HUMAN** Score: **116** Expect: **4e-008**  
**Calmodulin-like protein 3 (Calmodulin-related protein NB-1) (CaM-like protein) (CLP) - Homo sapiens**

Nominal mass ( $M_r$ ): **16880**; Calculated pI value: **4.30**  
 NCBI BLAST search of CALL3\_HUMAN against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **11%**

Matched peptides shown in **Bold Black**

**1** MADQLTEEQV TEFKEAFSLF DKDGDGCITT RELGTVMRSL GQNPTAEELR  
**51** DMMSEIDRDG NGTVDFPEFL GMMARKMKDT DNEEEIREAF RVFDKDGNGF  
**101** VSAAELRHVM TRLGKLSDE EVDEMIR**AAD TDGDGQVNYE EFVR**VLVSK

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
128 - 144	1885.80	1884.79	1884.81	-0.02	0	R.AADTDGDGQVNYEEFVR.V

**SPOT ID. 28*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**

Match to: **LEG7\_HUMAN** Score: **534** Expect: **6.4e-050**  
**Galectin-7 (Gal-7) (HKL-14) (PI7) (p53-induced protein 1) - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **15066**; Calculated pI value: **7.03**  
 NCBI BLAST search of **LEG7\_HUMAN** against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **88%**

Matched peptides shown in **Bold Black**

**1 MSNVPHKSSL PEGIRPGTVL RIRGLVPPNA SRFHVNLLCG EEQGSDAALH**  
**51 FNPRLDTSEV VENSKEQGSW GREERGPGVP FQRGQPFEVL IIASDDGFKA**  
**101 VVGDAQYHHE RHRLPLARVR LVEVGGDVQL DSVRIF**

Start	End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
8	21	1481.87	1480.86	1480.84	0.03	0	K.SSLPEGIRPGTVLR.I
22	32	1179.63	1178.62	1178.69	-0.07	1	R.IRGLVPPNASR.F
24	32	910.53	909.52	909.50	0.02	0	R.GLVPPNASR.F
33	54	2511.22	2510.21	2510.18	0.04	0	R.FHVNLLCGEEQGSDAALHFNPR.L(C)
55	65	1238.64	1237.63	1237.62	0.01	0	R.LDTSEVVFNSK.E
66	72	819.39	818.38	818.37	0.01	0	K.EQGSWGR.E
66	75	1233.59	1232.58	1232.55	0.03	1	K.EQGSWGREER.G
73	83	1271.67	1270.66	1270.64	0.02	1	R.EERGPGVPFQR.G
76	83	857.47	856.46	856.46	0.01	0	R.GPGVPFQR.G
84	99	1735.91	1734.90	1734.88	0.02	0	R.GQPFEVLIIASDDGFK.A
100	111	1399.71	1398.70	1398.68	0.02	0	K.AVVGDAQYHHFR.H
100	113	1692.87	1691.86	1691.84	0.03	1	K.AVVGDAQYHHFRHR.L
121	134	1485.82	1484.81	1484.78	0.03	0	R.LVEVGGDVQLDSVR.I

SPOT ID. 29*(MATRIX)*  
*(SCIENCES)* Mascot Search Results

## Protein View

Match to: **LAMB3\_HUMAN** Score: **653** Expect: **8e-062**  
**Laminin subunit beta-3 precursor (Laminin 5 beta 3) (Laminin B1k chain)**  
**(Kalinin B1 chain) - Homo s**

Nominal mass ( $M_r$ ): **129489**; Calculated pI value: **7.14**  
 NCBI BLAST search of LAMB3\_HUMAN against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **30%**

Matched peptides shown in **Bold Black**

1	MRPFFLLCFA	LPGLLHAQQA	CSRGACYPPV	GDLLVGRTRF	LRASSTCGLT
51	KPETYCTQYG	EWQMKCKCD	SR <b>QPHNYSH</b>	RVENVASSSG	PMRWWQSQND
101	VNPVSLQLDL	DRRFQLQEVN	MEFQGPMPAG	MLIERSSDFG	KTWR <b>VYQYLA</b>
151	<b>ADCTSTFPRV</b>	<b>RQGRPQSWQD</b>	<b>VRCQSLPQRP</b>	<b>NAR</b> LNNGGKVQ	LNLMDLVSGI
201	PATQSQKI <b>QE</b>	<b>VGEITNLRVN</b>	FTRLAPVPQR	<b>GYHPPSAYYA</b>	<b>VSQRLRQGSC</b>
251	<b>FCHGHADRCA</b>	PKPGASAGPS	TAVQVHDVCV	CQHNTAGPNC	ERCAPFYNNR
301	PWRPAEQGDA	HECQRDCDNG	HSETCHFDPA	VFAASQGAYG	GVCDNCRDHT
351	EGKNCER <b>CQL</b>	<b>HYFR</b> NRPGA	SIQETCISCE	CDPDGAVPGA	PCDPVTGQCV
401	CK <b>EHVQGERC</b>	DLCKPGFTGL	TYANPQGCHR	CDCNILGSRR	DMPCDEESGR
451	CLCLPNVVGP	KCDQCAPYHW	KLASGQGCEP	CACDPHNSLS	PQCNQFTGQC
501	PCRE <b>EGFGGLM</b>	<b>CSAAAIR</b> QCP	DR <b>TYGDVATG</b>	<b>CRACDCDFRG</b>	TEGPGCDKAS
551	GR <b>CLCRPGLT</b>	<b>GPR</b> CDQCQRG	YCNRYPVCVA	CHPCFQTYDA	DLREQALRFG
601	RLRNATASLW	SGPGLDRGL	ASRILDAK <b>SK</b>	<b>IEQIR</b> AVLSS	PAVTEQEVQA
651	VASAILSLRR	TLQGLQLDLP	LEETLSLPR	<b>DLES</b> LDRSFN	<b>GLLTMYQRKR</b>
701	EQFEK <b>ISSAD</b>	<b>PSGA</b> FRMLST	<b>AYE</b> QSAQAAQ	<b>QVSD</b> SSRLLD	QLRDSRREAE
751	RLVRQAGGGG	GTGSPKLVAL	<b>RLEM</b> SSLPDL	<b>TPT</b> FNKLCGN	SRQMACTPIS
801	CPGELCPQDN	GTACGSRCRG	VLPR <b>AGGA</b> FL	<b>MAGQ</b> VAEQLR	<b>GFNA</b> QLQRTR
851	QMIR <b>AAEESA</b>	<b>SQIQ</b> SSAQR	L ETQVSASR <b>SQ</b>	<b>MEED</b> VRRTRL	<b>LIQQ</b> VRDFLT
901	DPDTPDAATIQ	EVSEAVLALW	LPTDSATVLQ	<b>KMNEI</b> QAI	<b>RLPN</b> VDLVLS
951	<b>QTKQ</b> DIARAR	<b>RLQAE</b> AEEAR	SR <b>AH</b> AVEGQV	<b>EDV</b> VGNLRQ	TVALQEAQDT
1001	MQGTSRSLRL	IQDR <b>VAE</b> VQQ	<b>VLR</b> PAEKLV	SMTKQLGDFW	TRMEELRHQA
1051	RQQGAEAVQA	QQLAEGASEQ	ALSAQEGFER	IKQKYAELKD	<b>RLGQ</b> SSMLGE
1101	<b>QGAR</b> IQSVKT	EAEELFGETM	EMMDR <b>MKD</b> ME	<b>LELL</b> RGSQAI	<b>MLR</b> SADLTGL
1151	EKR <b>VEQ</b> IRDH	INGRVLYYAT	CK		

## SPOT ID 29, continued

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
73 - 81	1201.65	1200.64	1200.54	0.10	0	R.QPHNYSHR.V
145 - 159	1791.98	1790.97	1790.83	0.14	0	R.VYQYLAADCTSTFPR.V (C)
162 - 172	1356.79	1355.78	1355.67	0.11	0	R.QGRPQSWQDVR.C
173 - 183	1269.72	1268.71	1268.64	0.07	0	R.CQSLPQRPNAR.L
208 - 218	1271.80	1270.79	1270.69	0.10	0	K.IQEVGEITNLR.V
231 - 245	1708.97	1707.97	1707.84	0.13	0	R.GYHPPSAYYAVSQLR.L
246 - 258	1544.77	1543.76	1543.64	0.12	0	R.LQGSCFCHGADR.C 2 (C)
358 - 364	1023.56	1022.56	1022.48	0.08	0	R.CQLHYFR.N (C)
403 - 409	854.47	853.46	853.40	0.06	0	K.EHVQGER.C
504 - 517	1439.80	1438.79	1438.67	0.12	0	R.EGFGGLMCSAAAIR.Q (C)
523 - 532	1099.57	1098.56	1098.48	0.09	0	R.TYGDVATGCR.A (C)
553 - 563	1172.70	1171.69	1171.60	0.10	0	R.CLCRPGLTGPR.C
553 - 563	1286.75	1285.74	1285.64	0.10	0	R.CLCRPGLTGPR.C 2 (C)
629 - 635	873.58	872.57	872.51	0.06	1	K.SKIEQIR.A
681 - 687	847.49	846.48	846.41	0.07	0	R.DLESLDR.S
688 - 698	1329.77	1328.76	1328.65	0.11	0	R.SFNGLLTMYQR.K
688 - 699	1473.87	1472.87	1472.74	0.12	1	R.SFNGLLTMYQRK.R (M)
706 - 716	1107.64	1106.63	1106.54	0.09	0	K.ISSADPSGAFR.M
717 - 737	2258.24	2257.23	2257.03	0.21	0	R.MLSTAYEQSAQAAQVSDSSR.L
772 - 786	1708.97	1707.97	1707.84	0.13	0	R.LEMSSLPDLTPTFNK.L (M)
825 - 840	1618.96	1617.96	1617.83	0.13	0	R.AGGAFLMAGQVAEQLR.G
841 - 848	933.57	932.56	932.48	0.08	0	R.GFNAQLQR.T
855 - 869	1562.89	1561.88	1561.73	0.14	0	R.AAEESASQIQSSAQR.L
879 - 887	1149.63	1148.62	1148.52	0.10	1	R.SQMEEDVRR.T
888 - 896	1126.65	1125.65	1125.70	-0.05	1	R.TRLLIQQVR.D
890 - 896	869.62	868.61	868.55	0.06	0	R.LLIQQVR.D
932 - 941	1116.67	1115.66	1115.58	0.09	0	K.MNEIQAIAR.L
942 - 953	1326.82	1325.81	1325.76	0.05	0	R.LPNVDLVLSQTK.Q
962 - 970	1016.59	1015.59	1015.49	0.09	0	R.LQAEAEAR.S
973 - 988	1693.01	1692.00	1691.86	0.14	0	R.AHAVEGQVEDVVGNL.R.Q
1015 - 1027	1466.95	1465.95	1465.83	0.12	0	R.VAEVQQLRPAEK.L
1092 - 1104	1333.77	1332.77	1332.65	0.12	0	R.LGQSSMLGEQGAR.I
1126 - 1135	1277.76	1276.76	1276.65	0.11	1	R.MKDMELELLR.G
1126 - 1135	1309.76	1308.75	1308.64	0.11	1	R.MKDMELELLR.G 2 (M)
1136 - 1143	875.55	874.54	874.47	0.07	0	R.GSQAIMLR.S
1136 - 1143	891.47	890.46	890.46	-0.00	0	R.GSQAIMLR.S (M)
1153 - 1158	800.53	799.52	799.47	0.06	1	K.RVEQIR.D

**SPOT ID. 30*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**

Match to: **SPRC\_HUMAN** Score: **250** Expect: **1.6e-021**  
**SPARC precursor (Secreted protein acidic and rich in cysteine)**  
**(Osteonectin) (ON) (Basement-membran**

Nominal mass ( $M_r$ ): **34610**; Calculated pI value: **4.73**  
 NCBI BLAST search of **SPRC\_HUMAN** against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **29%**

Matched peptides shown in **Bold Black**

```

1 MRAWIFFLLC LAGRALAAPQ QEALPDETEV VEETVAEVTE VSVGANPVQV
51 EVGEFDDGAE ETEEEVVAEN PCQNHCKHG KVCELDEENNT PMCVCQDPTS
101 CPAPIGEFEK VCSNDNKTFD SSCHFFATKC TLEGTKKGHK LHLDYIGPCK
151 YIPPCLDSEL TEFPLMRDW LKNVLVTLYE RDEDNNLLTE KQKLRVKKIH
201 ENEKRLEAGD HPVELLARDF EKNYNMYIFP VHWQFGQLDQ HPIDGYLSHT
251 ELAPLRAPLI PMEHCTRFF ETCDLDNDKY IALDEWAGCF GIKQKDIDKD
301 LVI

```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
118 - 129	1447.59	1446.58	1446.62	-0.04	0	K.TFDSSCHFFATK.C (C)
141 - 150	1215.58	1214.58	1214.61	-0.04	0	K.LHLDYIGPCK.Y (C)
151 - 166	1949.92	1948.91	1948.96	-0.05	0	K.YIPPCLDSELTEFPLR.M (C)
167 - 172	848.41	847.40	847.44	-0.03	1	R.MRDWLK.N
173 - 181	1106.58	1105.57	1105.61	-0.04	0	K.NVLVTLYER.D
173 - 191	2278.10	2277.10	2277.15	-0.05	1	K.NVLVTLYERDEDNNLLTEK.Q
205 - 218	1575.82	1574.81	1574.85	-0.04	1	K.RLEAGDHPVELLAR.D
206 - 218	1419.72	1418.72	1418.75	-0.04	0	R.LEAGDHPVELLAR.D
257 - 268	1425.66	1424.65	1424.69	-0.04	0	R.APLIPMEHCTR.F (C)



SPOT ID. 31*{MATRIX}*  
*{SCIENCES}* Mascot Search Results**Protein View**

Match to: **TIMP1\_HUMAN** Score: **270** Expect: **1.6e-023**  
**Metalloproteinase inhibitor 1 precursor (TIMP-1) (Erythroid-potentiating activity) (Chevalier et al.) (Tissue in**

Nominal mass ( $M_r$ ): **23156**; Calculated pI value: **8.46**  
 NCBI BLAST search of TIMP1\_HUMAN against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **41%**

Matched peptides shown in **Bold Black**

```

1 MAPFEPLASG ILLLLWLIAP SRACTCVPPH PQTAFCNDDL VIRAKFVGTP
51 EVNQTTLYQR YEIKMTKMYK GFQALGDAAD IRFVYTPAME SVCGYFHRSH
101 NRSEEFLLIAG KLQDGLLHIT TCSEFVAPWNS LSLAQRRGFT KTYTVGCEEC
151 TVFPCLSIPC KLQSGTHCLW TDQLLQGSEK GFQSRHLACL PREPGLCTWQ
201 SLRSQIA

```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
71 - 82	1233.60	1232.59	1232.61	-0.02	0	K.GFQALGDAADIR.F
83 - 98	1963.86	1962.85	1962.88	-0.02	0	R.FVYTPAMESVCGYFHR.S (C)
83 - 98	1979.85	1978.85	1978.87	-0.02	0	R.FVYTPAMESVCGYFHR.S (C); (M)
142 - 161	2250.05	2249.05	2248.99	0.06	0	K.TYTVGCEECTVFPCLSIPCK.L (C)
162 - 180	2201.05	2200.05	2200.06	-0.01	0	K.LQSGTHCLWTDQLLQGSEK.G (C)
186 - 192	866.45	865.44	865.46	-0.02	0	R.HLACLPR.E (C)
193 - 203	1346.64	1345.63	1345.64	-0.02	0	R.EPGLCTWQSLR.S (C)

**SPOT ID. 32*****{MATRIX}*  
*{SCIENCES}* Mascot Search Results****Protein View**Match to: **S10A8\_HUMAN** Score: **122** Expect: **1e-008****Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A) (Migration inhibitory factor-rela**Nominal mass ( $M_r$ ): **10828**; Calculated pI value: **6.51**NCBI BLAST search of S10A8\_HUMAN against nrUnformatted sequence string for pasting into other applicationsTaxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **26%**Matched peptides shown in **Bold Black**

1 MLTELEKALN SIIDVYHKYS LIK**GNFHAVY RDDLKLEET ECPQYIRKKG**  
 51 ADVWFKELDI NTDGAVNFQE FLILVIKMGV AAHKKSHEES HKE

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
24 - 31	963.51	962.51	962.47	0.04	0	K.GNFHAVYR.D
24 - 35	1434.78	1433.78	1433.71	0.07	1	K.GNFHAVYRDDLK.K
36 - 47	1549.86	1548.85	1548.80	0.05	1	K.KLETECPQYIR.K (C)
37 - 47	1421.76	1420.75	1420.70	0.05	0	K.LLETECPQYIR.K (C)
37 - 48	1549.86	1548.85	1548.80	0.05	1	K.LLETECPQYIRK.K (C)

**SPOT ID. 33*****{MATRIX}*  
*{SCIENCES}* Mascot Search Results****Protein View**

Match to: **IL1RA\_HUMAN** Score: **322** Expect: **1e-028**  
**Interleukin-1 receptor antagonist protein precursor (IL-1ra) (IRAP) (IL1 inhibitor) (IL-1RN) (ICIL-**

Nominal mass ( $M_r$ ): **20042**; Calculated pI value: **5.83**  
 NCBI BLAST search of IL1RA\_HUMAN against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **30%**

Matched peptides shown in **Bold Black**

**1** MEICRGLRSH LITLLFLFLFH SETICRPSGR KSSKMQAFRI WDVNQKTFYL  
**51** **RNNQLVAGYL QGPNVNLEEK IDVVPIEPHA LFLGIHGGKM** CLSCVKSGDE  
**101** **TRLQLEAVNI TDLSENRKQD** KRFAFIRSDS GPTTSFESAA CPGWFLCTAM  
**151** EADQPVSLTN MPDEGVMVTK FYFQEDE

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
52 - 70	2100.09	2099.08	2099.06	0.01	0	R.NNQLVAGYLQGPNVNLEEK.I
71 - 89	2012.14	2011.13	2011.13	0.00	0	K.IDVVPIEPHALFLGIHGGK.M
103 - 117	1714.90	1713.90	1713.89	0.01	0	R.LQLEAVNITDLSENR.K
103 - 118	1842.98	1841.98	1841.98	-0.01	1	R.LQLEAVNITDLSENRK.Q

**SPOT ID. 34*****{MATRIX}***  
***{SCIENCES}*** Mascot Search Results**Protein View**

Match to: **RCN1\_HUMAN** Score: **571** Expect: **1.3e-053**  
**Reticulocalbin-1 precursor - Homo sapiens (Human)**

Nominal mass ( $M_r$ ): **38866**; Calculated pI value: **4.86**  
 NCBI BLAST search of **RCN1\_HUMAN** against nr  
 Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C), Oxidation (M)  
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
 Sequence Coverage: **37%**

Matched peptides shown in **Bold Black**

```

1 MARGGRGRRL GLALGLLLAL VLAPRVLRAK PTVRKERVVR PDSELGERPP
51 EDNQSFYQYDH EAFLGKEDSK TFDQLTPDES KERLGKIVDR IDNDGDGFVT
101 TEELKTWIKR VQKRYIFDNV AKVWKDYDRD KDDKISWEEY KQATYGYLLG
151 NPAEFHDSSD HHTFFKMLPR DERREFAADL NGDLTATREE FTAFHLPEEF
201 EHMKEIIVVLE TLEDIDKNGD GFVDQDEYIA DMFSHEENGP EPDWVLSERE
251 QFNEFRDLNK DGKLDKDEIR HWILPQDYDH AQAEARHLVY ESDKNKDEKL
301 TKEEILENWN MFVGSQATNY GEDLTKNHDE L

```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
71 - 83	1565.73	1564.72	1564.74	-0.01	1	K.TFDQLTPDESKER.L
87 - 105	2136.03	2135.02	2135.04	-0.02	1	K.IVDRIDNDGDGFVTTEELK.T
91 - 105	1652.75	1651.75	1651.76	-0.01	0	R.IDNDGDGFVTTEELK.T
114 - 122	1125.59	1124.58	1124.60	-0.01	1	K.RYIFDNVAK.V
123 - 129	981.45	980.44	980.47	-0.03	1	K.VWKDYDR.D
135 - 141	954.44	953.43	953.45	-0.02	0	K.ISWEEYK.Q
177 - 188	1217.60	1216.59	1216.60	-0.01	0	K.AADLNGDLTATR.E
189 - 204	2020.90	2019.89	2019.90	-0.01	0	R.EEFTAFHLPEEFEHMK.E
189 - 204	2036.90	2035.89	2035.90	-0.00	0	R.EEFTAFHLPEEFEHMK.E (M)
250 - 256	969.44	968.43	968.44	-0.00	0	R.EQFNEFR.D
250 - 260	1439.67	1438.67	1438.68	-0.02	1	R.EQFNEFRDLNK.D
264 - 270	888.46	887.46	887.47	-0.02	1	K.LDKDEIR.H
271 - 286	1949.92	1948.92	1948.92	-0.00	0	R.HWILPQDYDHAQAEAR.H
287 - 294	990.48	989.47	989.48	-0.01	0	R.HLVYESDK.N

**SPOT ID. 35*****(MATRIX)*  
*(SCIENCES)* Mascot Search Results****Protein View**Match to: **LAMA3\_HUMAN** Score: **122** Expect: **1e-008****Laminin subunit alpha-3 precursor (Epiligrin 170 kDa subunit) (E170) (Nicein subunit alpha) - Homo**Nominal mass ( $M_r$ ): **189187**; Calculated pI value: **8.41**NCBI BLAST search of **LAMA3\_HUMAN** against nrUnformatted [sequence string](#) for pasting into other applicationsTaxonomy: [Homo sapiens](#)

Variable modifications: Carbamidomethyl (C), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **2%**Matched peptides shown in **Bold Black**

**1** MGWLWIFGAA LGQCLGYSSQ QQRVPFLQPP GQSQLQASYV EFRPSQGCSP  
**51** GYYRDHKGLY TGRCVPCNCN GHSNQCDGS GICVNCQHNT AGEHCERCQE  
**101** GYYGNAVHGS CRACPCPTN SFATGCVVNG GDVRCCKAG YTGTQCERCA  
**151** PGYFGNPQKF GGSCQPCSCN SNGQLGSCHP LTGDCINQEP KDSSPAEECD  
**201** DCDCSCVMTLL NDLATMGEQL RLVKSQQLQGL SASAGLLEQM RHMETQAKDL  
**251** RNQLLNYSRA ISNHGSKIEG LERELTDLNQ EFETLQEKAQ VNSRKAQTLN  
**301** NNVNRRATQSA KELDVKIKNV IRNVHILLKQ ISGTDGEGNN VPSGDFSREW  
**351** AEAQRMMREL RNRNFGKHLR EAEADKRESQ LLLNRIRTWQ KTHQGENNGL  
**401** ANSIRDSLNE YEAKLSDLRA RLQEAQAQAK QANGLNQENE RALGAIQRQV  
**451** KEINSLQSDF TKYLTTADSS LLQTNIALQL MEKSQKEYEK LAASLNEARQ  
**501** ELSDKVRELS RSAGKTSLVE EAEKHARSLQ ELAKQLEEIK RNASGDELVR  
**551** CAVDAATAYE NILNAIKAAE DAANRAASAS ESALQTVIKE DLPRKAKTSL  
**601** SNSDKLLNEA KMTQKKLKQE VSPALNNLQQ TLNIVTVQKE VIDTNLTTLR  
**651** DGLHGIQRGD IDAMISSAKS MVRKANDITD EVLDGLNPIQ TDVERIKDITY  
**701** GRTQNEDEFK ALTDADNSVN KLTNKLPLDW RKIESINQQ LPLGNISDNM  
**751** DRIRELIQQA RDAASKVAVP MRFNGKSGVE VRLPNDLEDL KGYTSLSLFL  
**801** QRPNSRENGG TENMFVMYLG NKDASRDYIG MAVVDGQLTC VYNLGDREAE  
**851** LQVDQILTKS ETKEAVMDRV KFQRIYQFAR LNYTKGATSS KPETPGVYDM  
**901** DGRNSNTLLN LDPENVVFYV GGYPPDFKLP SRLSFPYK CIELDDLNEN  
**951** VLSLYNFKKT FNLNTEVEP CRRRKEESDK NYFEGTGYAR **VPTQPHAPIP**  
**1001** **TFGQTIQTTV DRGLLEFFAEN GDRFISLNI** DGKLMVRYKL NSELPKERGV  
**1051** GDAINNGRDH SIQIKIGKLQ KRMWINVDVQ NTIIDGEVFD FSTYYLGGIP  
**1101** IAIRERFNIS TPAFRGCMKN LKKTSGVVRL NDTVGVTKK SEDWKLVRSA  
**1151** SFSRGGQLSF TDLGLPPTDH LQASFGFQTF QPSGILLDHQ TWTRNLQVTL  
**1201** EDGYIELSTS DSGGPIFKSP QTYMDGLLHY VSVISDNSGL RLLIDDQLLR  
**1251** NSKRLKHISS SRQSLR**LGG** **NFEGCISNVF VQR**LSLSPEV LDLTNSLSLKR  
**1301** DVSLGGCSLN KPPFLMLLKG STRFNKTKTF RINQLLQDTP VASPRSVKVV  
**1351** QDACSPPKT QANHGALQFG DIPSHLLFK LPQELKPRS QFAVDMQTTS  
**1401** SRGLVFHTGT KNSFMALYLS KGRLVFALGT DGKKLRIKSK EKCNDGKWH  
**1451** VVFGHDGEKG RLVVDGLRAR EGSLPGNSTI SIRAPVYLGSP PSFGPKSLP  
**1501** TNSFVGCLKN FQLDSKPLYT PSSSFGVSSC LGGPLEKGIY FSEEGGHVVL  
**1551** AHSVLLGPEF KLVFSIRPRS LTGILIHIGS QPGKHLVCVYL EAGKVTASMD  
**1601** SGAGGTSTSV TPKQSLCDGQ WHSVAVTIKQ HILHLELTD SSYTAGQIPF  
**1651** PPASTQEPLH LGGAPANLTT LRIPVWKSFF GCLRNIHVNH IPVPVTEALE  
**1701** VQGPVSLNGC PDQ

## SPOT ID. 35, continued

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss Sequence
991 - 1012	2404.24	2403.23	2403.25	-0.02	0 R.VPTQPHAPIPTFGQTIQTTVDR.G
1013 - 1023	1238.61	1237.60	1237.61	-0.01	0 R.GLLFFAENGDR.F
1267 - 1283	1883.89	1882.89	1882.90	-0.01	0 R.LGGSNFEGCISNVFVQR.L (C)

## IPA<sup>®</sup> Summary of Analysis

INGENUITY<sup>®</sup>  
S Y S T E M S

Analysis Name: cancer all - 2012-06-27  
 Analysis Creation Date: 2012-06-27  
 Build version: 140500  
 Content version: 12710793 (Release Date: 2012-05-07)

### Analysis settings

#### View

Reference set: Ingenuity Knowledge Base (Genes + Endogenous Chemicals)  
 Relationship to include: Direct and Indirect  
 Includes Endogenous Chemicals  
 Optional Analyses: My Pathways My List

#### Filter Summary:

Consider only relationships where  
 confidence = Experimentally Observed

### Top Networks

ID	Associated Network Functions	Score
1	Cancer, Gastrointestinal Disease, Cellular Movement	48
2	Cellular Growth and Proliferation, Post-Translational Modification, Cell-To-Cell Signaling and Interaction	15
3	Embryonic Development, Tissue Development, Tissue Morphology	12

## Summary of Analysis - cancer all - 2012-06-27

## Top Bio Functions

## Diseases and Disorders

Name	p-value	# Molecules
Cancer	5.40E-09 - 2.16E-02	23
Gastrointestinal Disease	5.40E-09 - 2.08E-02	18
Endocrine System Disorders	1.05E-07 - 1.91E-02	10
Hereditary Disorder	3.63E-07 - 2.08E-02	18
Immunological Disease	1.65E-06 - 1.80E-02	12

## Molecular and Cellular Functions

Name	p-value	# Molecules
Cellular Movement	1.13E-10 - 2.21E-02	20
Cell Death	2.11E-07 - 2.25E-02	19
Cellular Development	1.18E-06 - 1.87E-02	21
Cellular Growth and Proliferation	1.18E-06 - 2.00E-02	21
Carbohydrate Metabolism	2.94E-05 - 2.25E-02	4

## Physiological System Development and Function

Name	p-value	# Molecules
Immune Cell Trafficking	1.76E-05 - 2.21E-02	8
Tissue Development	4.86E-05 - 2.25E-02	17
Hematological System Development and Function	5.37E-05 - 2.21E-02	11
Tissue Morphology	5.74E-05 - 2.25E-02	14
Tumor Morphology	3.94E-04 - 1.73E-02	9



## Summary of Analysis - cancer all - 2012-06-27

## Top Canonical Pathways

Name	p-value	Ratio
RhoGDI Signaling	3.72E-03	3/199 (0.015)
Glioma Invasiveness Signaling	4.59E-03	2/60 (0.033)
Leukocyte Extravasation Signaling	4.73E-03	3/199 (0.015)
Glutathione Metabolism	7.37E-03	2/89 (0.022)
Signaling by Rho Family GTPases	7.53E-03	3/253 (0.012)

## Top Molecules

## Fold Change up-regulated

Molecules	Exp. Value	Exp. Chart
CDH2	↑1000000.000	
CTSD	↑1000000.000	
TIMP2 (includes EG:21858)	↑55.543	
FSTL1	↑17.118	
HNRNPA2B1*	↑15.231	
PRDX1	↑8.409	
HSPE1	↑7.614	
ANP32B	↑6.993	
PCNA*	↑5.556	
MMP7	↑4.491	

## Fold Change down-regulated

Molecules	Exp. Value	Exp. Chart
RCN1 (includes EG:19672)	↓-1000000.000	

## Appendix B

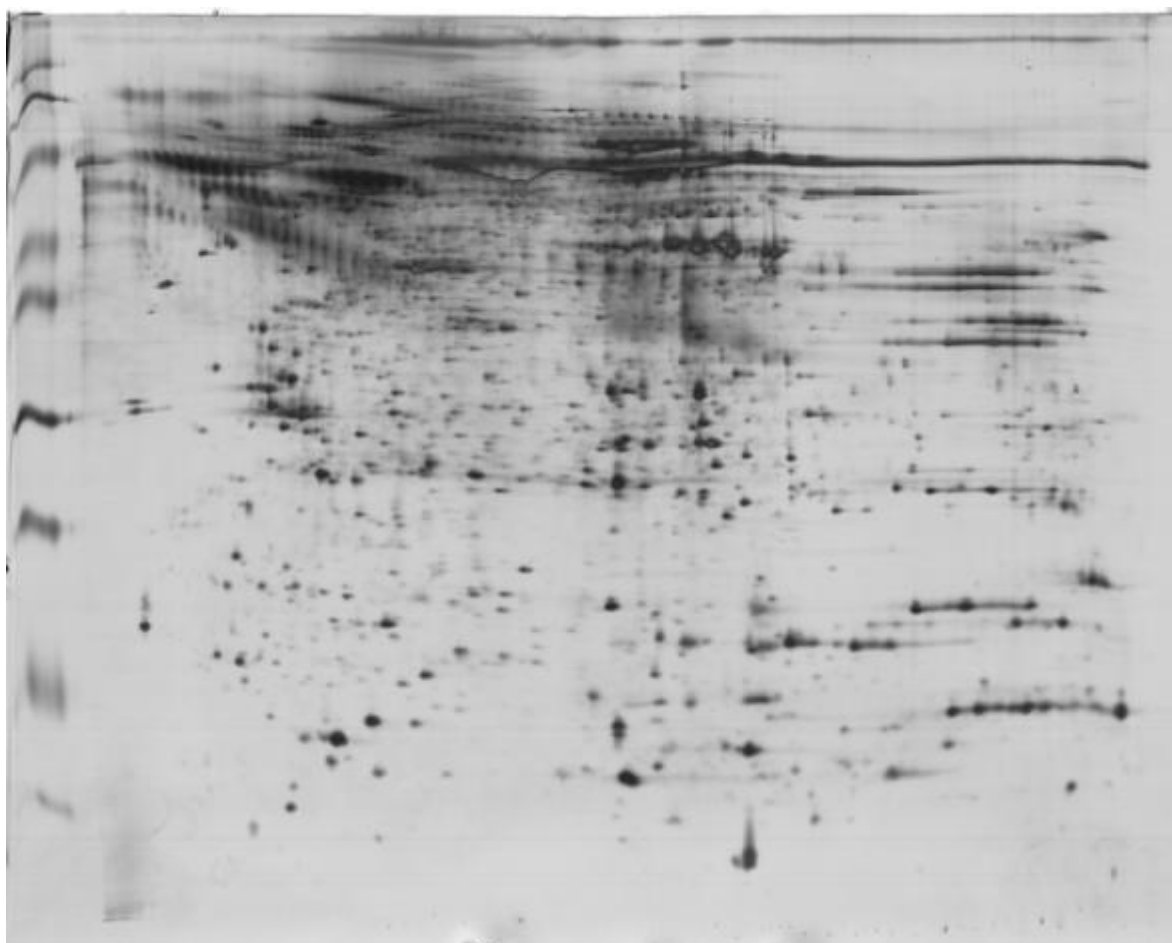
### Summary of Analysis - cancer all - 2012-06-27

LAMA3	+-1000000.000
IL1RN	+-1000000.000
S100A8	+-48.362
TIMP1	+-14.294
SPARC	+-8.764
LAMB3	+-7.423
LGALS7/LGALS7B	+-6.755
CALML3	+-5.716
SERPINB1	+-4.251

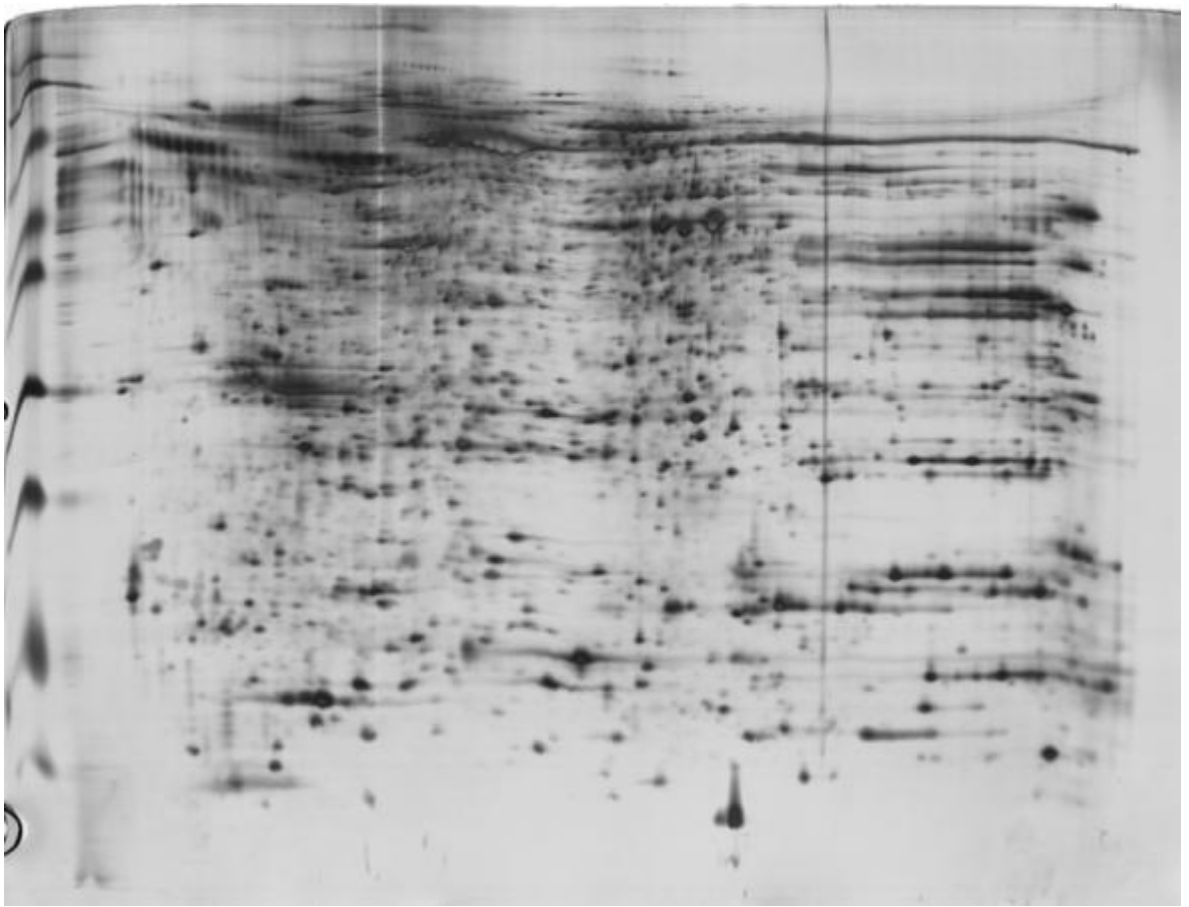
### Top Transcription Factors

Transcription Regulator	p-value of overlap	Predicted Activation State
MYC	1.49E-06	
CTNNB1	3.18E-04	
HTATIP2	6.22E-04	
SP3	1.75E-03	
MLL2	1.98E-03	

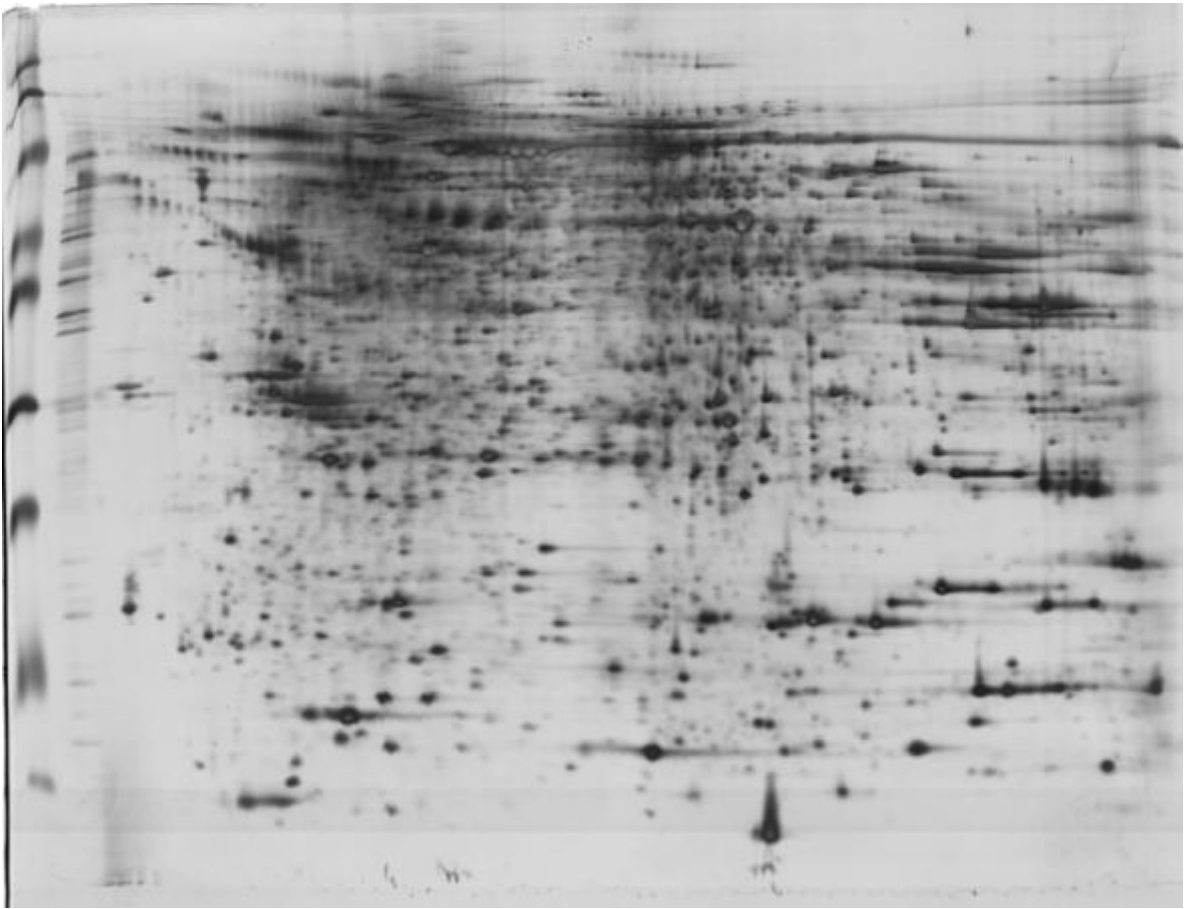
A representative gel image for each different studied cell lines and primary cultures:



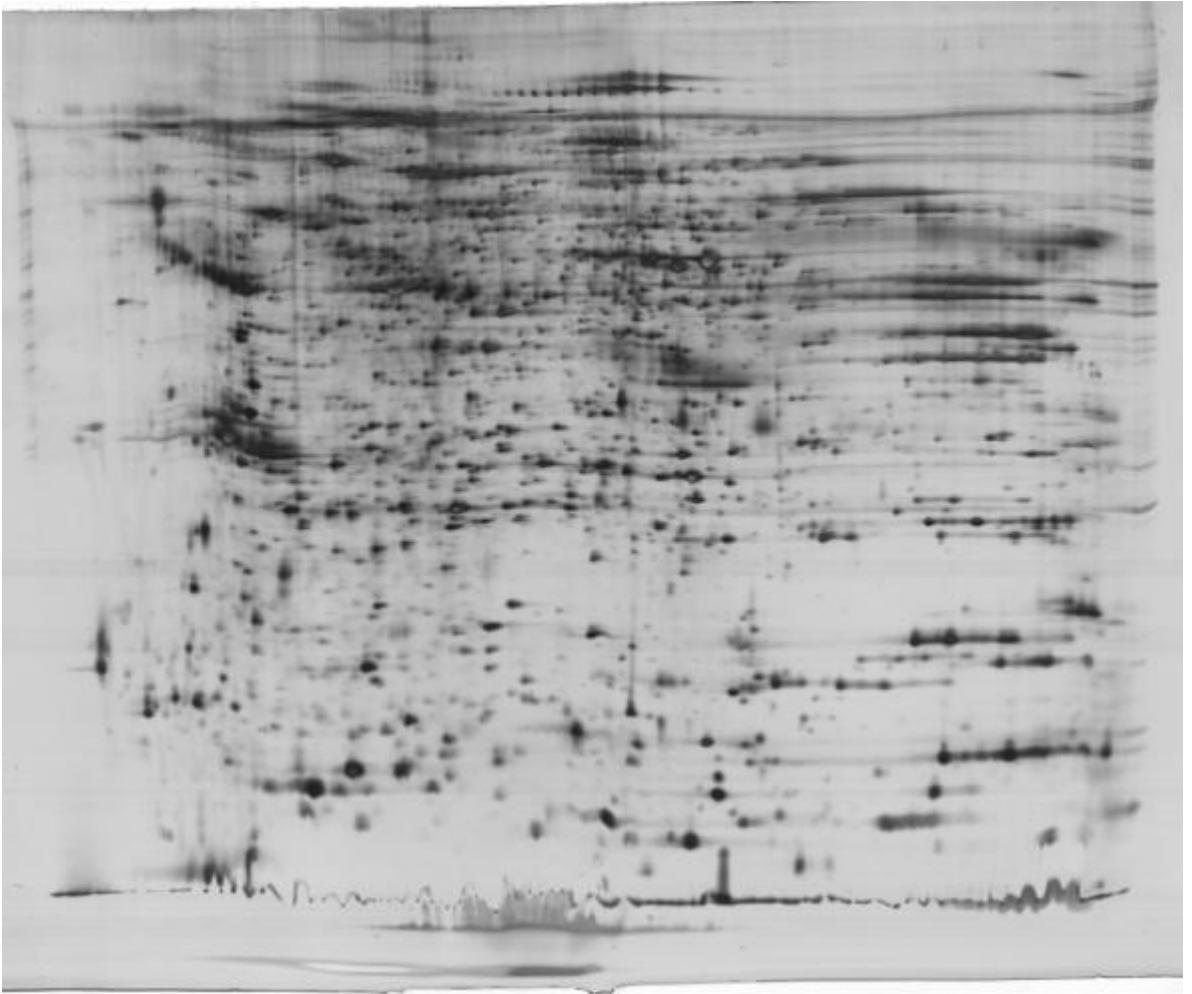
**48T**



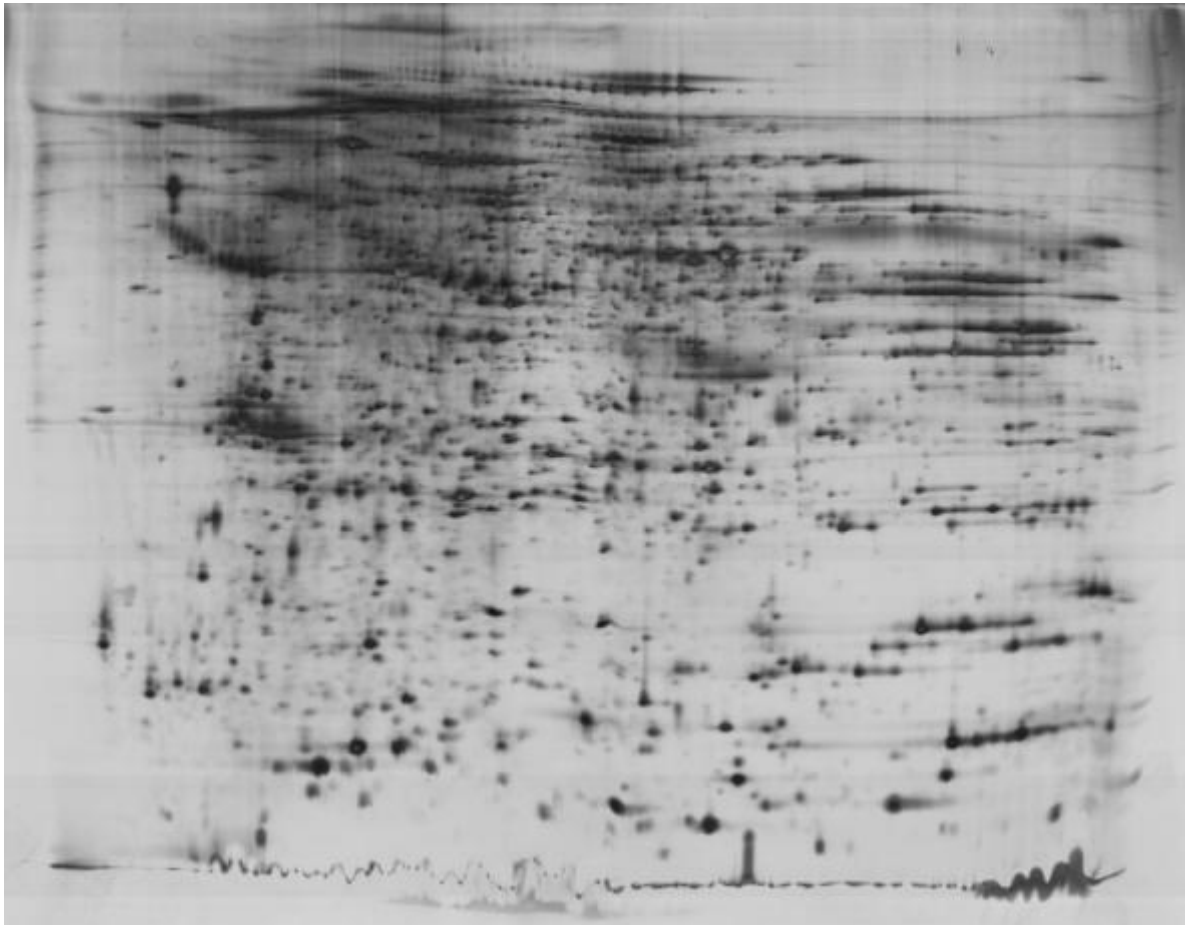
153T



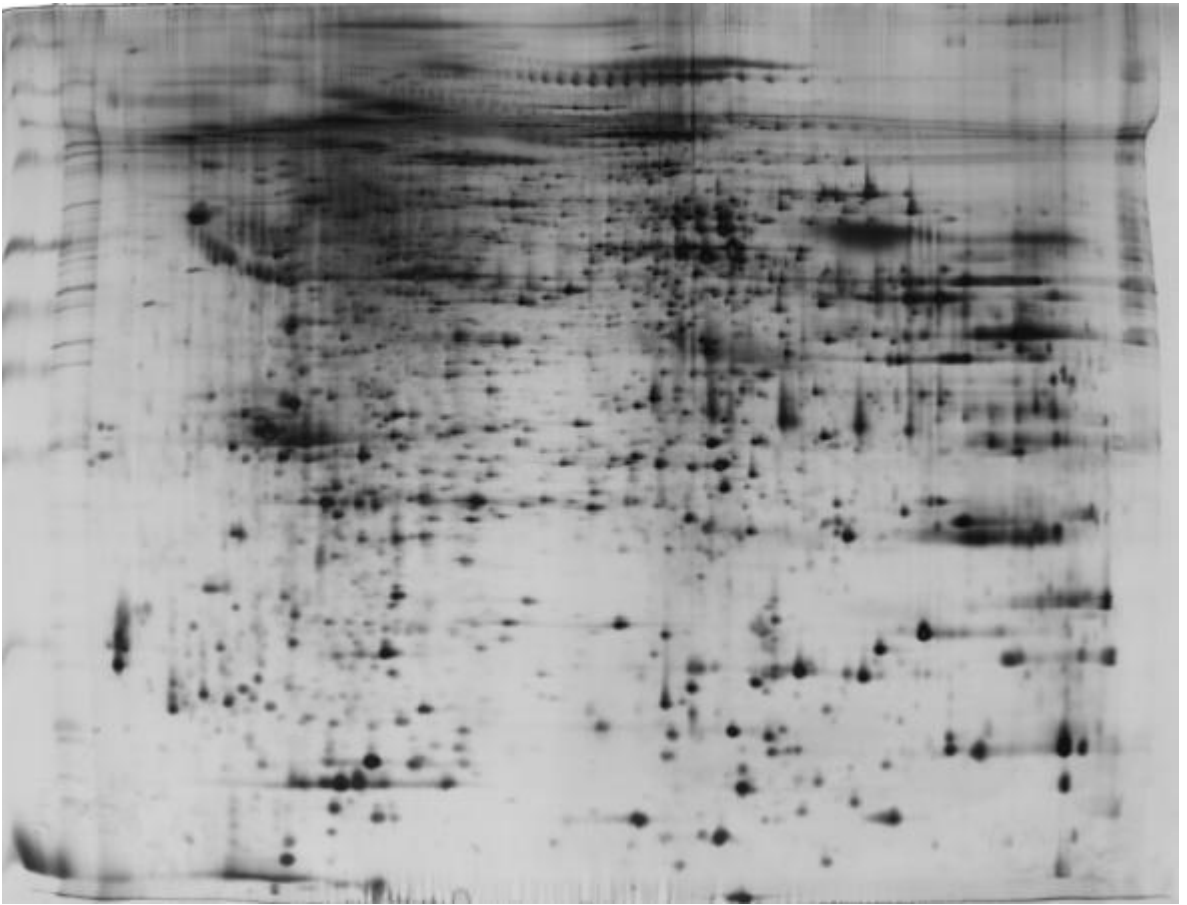
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316N

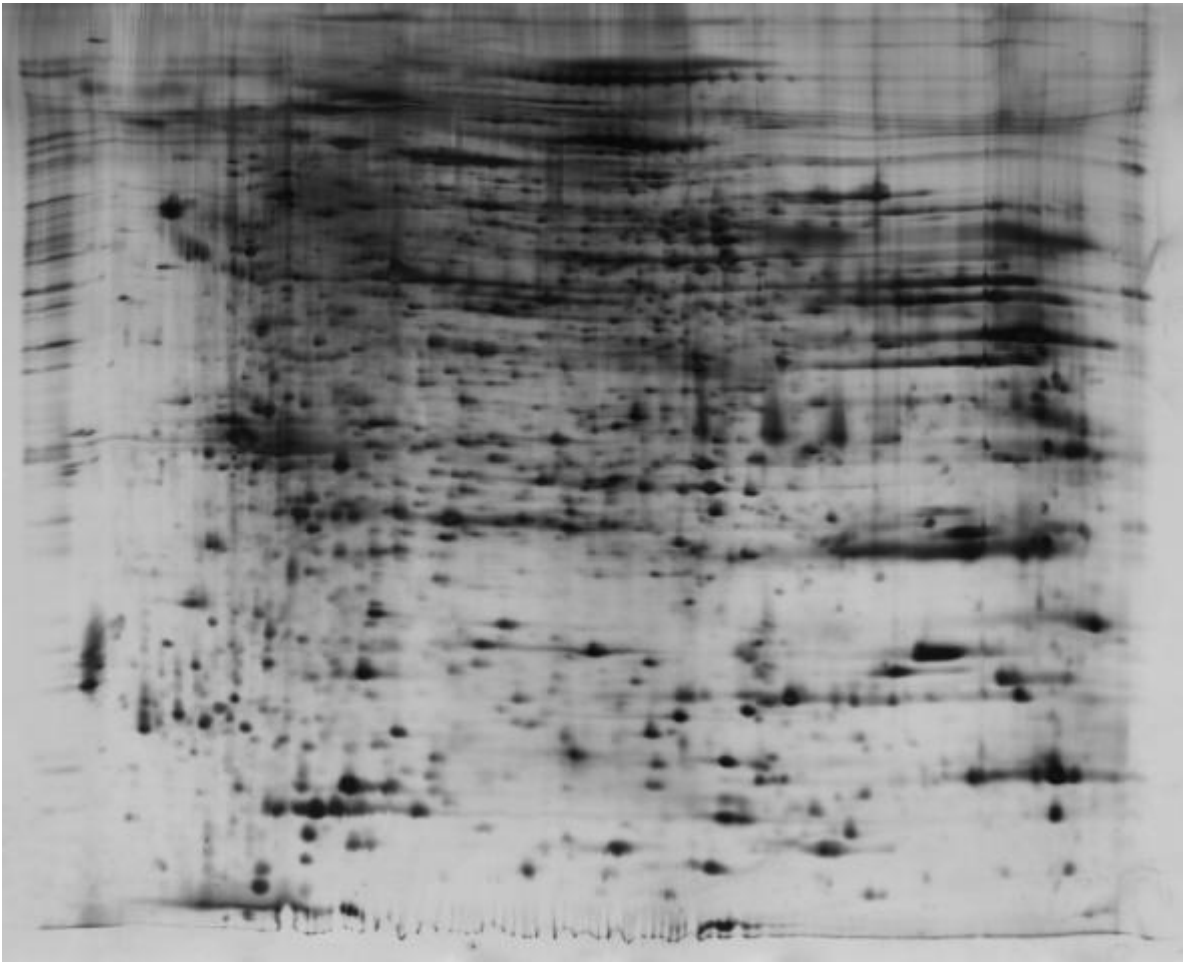


317N



322N





326N