PROTEOMIC ANALYSIS OF URINARY PROTEINS FROM PATIENTS WITH OVARIAN CANCER AND CERVICAL CANCER

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ABSTRACT

Diagnosis of ovarian carcinoma is in urgent need for new complementary biomarkers for early stage detection. In order to find an alternative procedure to replace an uncomfortable conventional method in detecting cervical cancer, a proteomic approach in screening the urinary proteins were employed. Proteins that are aberrantly excreted in the urine of cancer patients are excellent biomarker candidates for development of new non-invasive protocol for early diagnosis and screening purposes. In the present study, urine samples from patients with ovarian carcinoma and cervical cancer were analysed by two-dimensional gel electrophoresis (2-DE) and the profiles generated were compared to those similarly obtained from age-matched cancer negative women. These samples were also subjected to SELDI-TOF-MS as a complimentary technique for 2-DE especially on screening of aberrantly expressed of low molecular weight proteins.

Significant reduced levels of CD59, kininogen-1 and a 39 kDa fragment of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and enhanced excretion of a 19 kDa fragment of albumin, were detected in the urine of patients with ovarian carcinoma compared to the control subjects. These proteins, with exception of kininogen-1, were also detected in the urine of patients with cervical cancer as compared to the control subjects. The different altered levels of the proteins were confirmed by Western blotting using antisera and a lectin that bind to the respective proteins. When the samples were analysed with SELDI-TOF-MS, one protein peak with *m/z* of 15802 was detected in ovarian cancer cohort, but not in cervical cancer and control. The peaks *m/z* 7528.78 and *m/z* 8828.8 were found to be significantly absent in ovarian cancer and cervical cancer, respectively. Interestingly, protein peak at *m/z* 15802 with a *p*-value less than 0.05 had a potential to be a good biomarker with 100% sensitivity and 89.4% specificity for the learning set obtained from the classification tree in the Biomarker Pattern Software (BPS).

This study clearly demonstrated that the combined technology of 2-DE and SELDI-TOF-MS is effective in distinguishing urinary protein expression between control, ovarian carcinoma and cervical cancer cohorts. The identified proteins CD59, kininogen-1 and fragments of ITIH4 and albumin may be used as complementary biomarkers in the development of new non-invasive protocols for diagnosis and screening of ovarian carcinoma and cervical cancer. The identified peaks may be candidate biomarkers for early detection and could be utilised to distinguish between ovarian and cervical cancer in the future. Furthermore, the classification tree generated by the Biomarker Pattern Software (BPS), has a potential to be used in classifying the three cohorts.

ABSTRAK

Penanda pelengkap biologi bagi ujian diagnosis kanser ovari diperlukan dengan segera terutama bagi pengesanan peringkat awal penyakit ini. Bagi mengganti prosedur konvensional yang tidak menyelesakan dalam mengecam kanser servik, perkembangan dari kaedah lain seperti pendekatan proteomik dalam saringan terhadap protein di dalam air kencing dilakukan. Protein yang dikumuhkan secara aberan dalam air kencing penyakit kanser merupakan calon penanda biologi yang sangat baik dalam membangunkan protokol baru yang tidak invasif untuk diagnosis awal dan tujuan pemeriksaan. Dalam kajian ini, sampel air kencing daripada pesakit dengan karsinoma ovari dan kanser pangkal rahim telah dianalisis oleh elektroforesis gel dua dimensi (2-DE) dan profil yang dijana dibandingkan dengan wanita kontrol yang mempunyai padanan umur yang sama. Sampel ini juga telah digunakan dalam analisa menggunakan SELDI-TOF-MS sebagai teknik komplimentari bagi 2-DE terutama bagi saringan berat molekul protein rendah yang aberan.

Tahap CD59, kininogen-1, serpihan 39 kDa alpha-trypsin inhibitor heavy chain H4 (ITIH4) yang didapati berkurang secara signifikan, serta peningkatan serpihan 19 kDa albumin telah dikesan dalam air kencing pesakit karsinoma ovari berbanding wanita kontrol. Protein-protein tersebut kecuali kininogen-1 ini juga dikesan dalam air kencing kanser servik apabila dibandingkan dengan wanita kontrol. Tahap protein yang berbeza dari kebiasaan di dalam air kencing telah disahkan oleh kaedah Western blotting dengan menggunakan antisera dan lektin yang mengikat protein masing-masing. Apabila sampel dianalisa dengan SELDI-TOF-MS, satu puncak protein pada *m/z* 15802 dikesan dalam pesakit kanser ovari tetapi tidak di dalam kanser servik dan wanita kontrol. Puncak protein pada *m/z* 7528.78 dan *m/z* 8828.8 didapati tiada secara signifikan di dalam kedua-dua jenis kanser. Apa yang menarik ialah, puncak pada *m/z* 15802 dengan *p*-value kurang dari 0.05 mempunyai potensi untuk menjadi penanda bio yang baik dengan pencapaian 100% sensitif

dan 89.4% spesifik dari set pembelajaran yang diperoleh melalui kaedah klasifikasi di dalam Perisian Corak Biomarker (BPS).

Kajian ini jelas menunjukkan bahawa gabungan teknologi 2-DE dan SELDI-TOF-MS berkesan dalam memprofilkan protein yang berbeza dalam air kencing wanita kontrol, karsinoma ovari dan kanser servik. Protein CD59, kininogen-1, serpihan ITIH4 dan serpihan albumin dikenalpasti boleh digunakan sebagai pelengkap penanda biologi dalam pembangunan protokol baru yang tidak invasif bagi tujuan diagnosis dan pemeriksaan karsinoma ovari atau kanser pangkal rahim. Puncak protein yang dikenalpasti juga boleh dipertimbangkan sebagai calon protein bagi pengesanan awal dan membezakan antara karsinoma ovari dan kanser pangkal rahim pada masa akan datang. Tambahan juga, pokok klasifikasi yang dihasilkan oleh Perisian Corak biomarker (BPS), mempunyai potensi untuk digunakan dalam mengklasifikasikan ketiga kohort yang dikaji.

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ABBREVIATION

A_{280}	Absorbance at 280 nm
cm	centimeters
Da	Dalton(s)
ºC	Degrees celcius
2-DE	Two-dimensional gel electrophoresis
DTT	Dithiothreitol
DVS	Divinylsulfone
EDTA	Ethylenediaminetetraacetic acid
e.g	Exampli gratia (for instance)
et al.	Et alia (and others)
etc	Et ceteara (and so forth)
g	gram
HCl	Hydrochloric acid
hr	hour(s)
i.e.	Id Est (That is)
IEF	Isoelectric focusing
Ig	Immunoglobulin
IPG	Immobilised pH gradient
kDa	kilodalton(s)
L	Litre
M	Molar
mg	milligram
min	minute(s)
ml	mililitre

mM	milimolar
mmol	Milimole(s)
M_r	Relative molecular mass
Nm	nanometer(s)
PBS	Phosphate-buffered saline
%	Percentage
pI	Isoelectric point
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethyldiamine
Tris	Tris-(hydroxymethyl) aminomethane
V	Voltage
v/v	Volume over volume
w/v	Weight over volume
x g	Acceleration due to gravity
μg	microgram(s)
μl	microlitre(s)

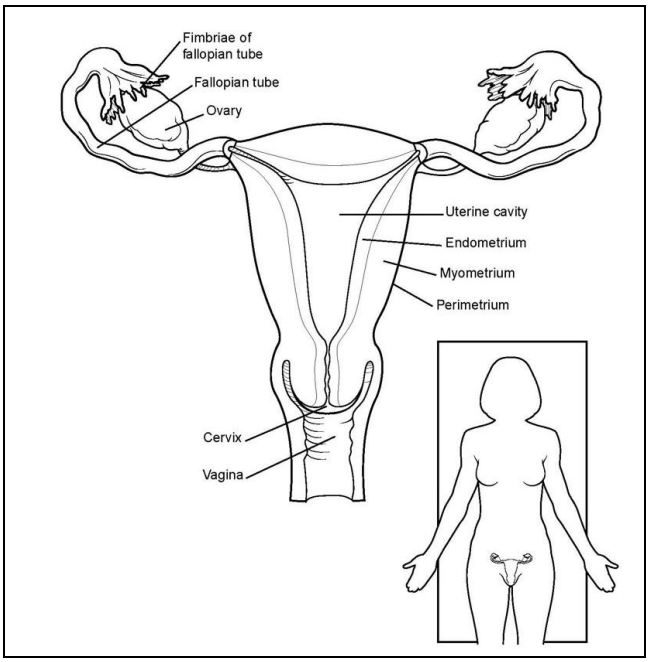
Chapter 1
INTRODUCTION

1.1 Female reproductive system

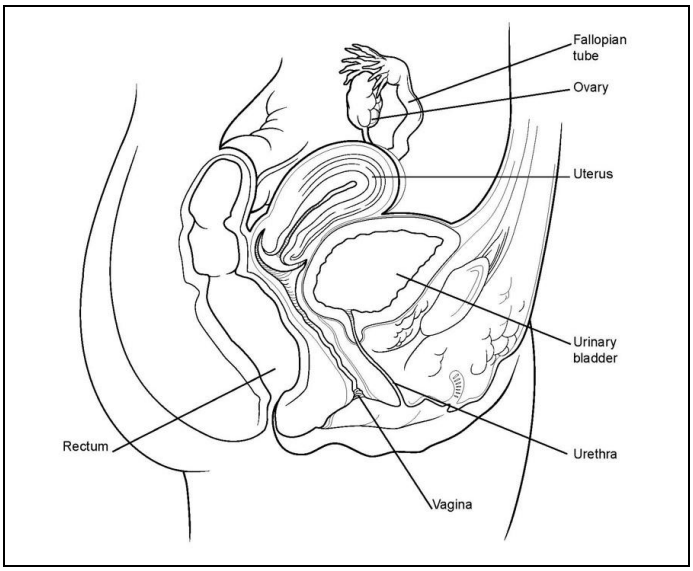
The purpose of the male and female reproductive systems is to continue the human species by the production of offspring. This is achieved through production of gametes, that is, sperm and egg cells, and ensures the union of gametes in fertilization following sexual intercourse (Scanlon & Sanders, 2007).

The female reproductive organs found inside the body includes the vagina, uterus, ovaries, and fallopian tubes (Figure 1). The uterus can be divided into two parts called cervix and corpus. The cervix is the lower part of the uterus that opens into the vagina whereas the corpus is at the upper part where the structure can easily expand to hold a developing baby (Chung, 2000). A channel through the cervix allows sperm to enter and menstrual blood to exit. The ovaries are oval-shaped glands that are located on either side of the uterus. The ovaries produce eggs (ova) and also main female sex hormones which are released into the bloodstream (MacLennan *et al.*, 1991). The clitoris, labia and a numeral of glands are all together known as vulva which is part of vagina that is found externally.

According to the American Academy of Pediatrics, American College of Obstetricians and Gynecologists in 2006, the length of the reproductive or also known as menstrual cycle is usually 24-35 days. The main function of the female reproductive system is to give women the ability to produce ova to be fertilised, space and conditions for a fetus to grow. This female reproductive system is important to allow sperm from a man to meet the ova of a woman during the sexual intercourse. The lining of the uterus is prepared to receive a fertilised egg during the time where the ova is developed and matured. It is shed and expelled from the body if a fertilised egg is not implanted into the uterus. This bleeding process is also known as menstruation.



Anterior view



Saggital section

Figure 1: Location of the female reproductive system in women

(Adapted with permission from http://emedicine.medscape.com assessed on 1 February 2012)

1.1.1 Hormones in female reproductive cycle

The activity of the female reproductive system is controlled by hormones released by the brain and the ovaries. There are five main hormones that control the reproductive cycle such as gonadotrophin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestrogen and progesterone (Lobo, 2007). There are around 20 immature ova begin to develop in the ovaries during the last few days of the menstrual cycle (MacLennan *et al.*, 1991). FSH and LH encourage the growth of these ova and as they grow, the ova also start to release increasing amount of oestrogen. The amount of oestrogen produced reduces the amount of FSH released which also prevents too many ova growing at the same time. As this is happening in the ovaries, the estrogen produced also stimulates the repair of the lining uterus.

The mature ovum from the ovary is then released into the pelvis. At this moment in the cycle, oestrogen levels are high. Previously, medium level of oestrogen reduced the amount of FSH and LH released. The high level of oestrogen is the signal for more FSH and LH to be released. LH causes the ovum to burst through the outer layer of the ovary where then the ovum is swept into the uterine tubes (Losos *et al.*, 2002). The cells remaining when the ovum leaves the ovary become the corpus luteum. This group of cells is able to produce several different hormones including progesterone and oestrogen (Weschler, 2002). These hormones encourage the growth and maturation of the lining of the uterus.

The next event will depends whether the ovum is fertilised by a sperm. If the ovum is fertilised, the corpus luteum will continue to produce hormone. A hormone called human chorionic gonadotropin (hCG) is produced by the cells covering the embryo will stop the corpus luteum from breaking down (Tay *et al.*, 2000). This is the hormone detected in the pregnancy test kit (Waddell *et al.*, 2006). If the ovum is not fertilized, the corpus luteum

can only live for another two weeks. It releases less of its hormones as it begins to breakdown. The levels of GnRH, FSH and LH are no longer being controlled as the levels of progesterone and oestrogen go down. Thus, these hormones increase and new ova begin to develop, which means the starting of a new cycle. In the uterus, the decrease in progesterone stimulates the release of chemicals that eventually cause the lining of the uterus to die off. This is the blood flow experienced during menstruation. This process will happen over and over again until women start experiencing menopause (Losos *et al.*, 2002). Any disturbance that occurred in the female reproductive system can lead to variety of disorders including infection, disorder of menstruation, pain (Sperrof, 2005) and malignancies. Figure 2 displays the location of the ovarian and cervical cancer that occurrs in the female reproductive system.

1.2 Ovarian carcinoma

Gynaecological cancer is a group of cancers that affect the tissues and organs of the female reproductive system. Each type of cancer is named after the organ it originates and they includes cervical cancer, ovarian cancer, uterine cancer, vaginal cancer and vulvar cancer (Lisa, 2010). Ovarian cancer is the fourth most common cancer affecting women in Malaysia (Lim *et al*., 2003). A critical factor in the elevated mortality associated with ovarian cancer is the lack of disease-specific symptoms.

Although the cure rate for stage I disease is usually greater than 90%, the five year survival rate for patients with clinically advanced ovarian cancer is only 15-20% (Holschneider *et al.*, 2000). Therefore, improved screening methodologies aimed at detecting ovarian cancer at its earliest stage have the potential to result in considerable improvement in overall survival of this disease.

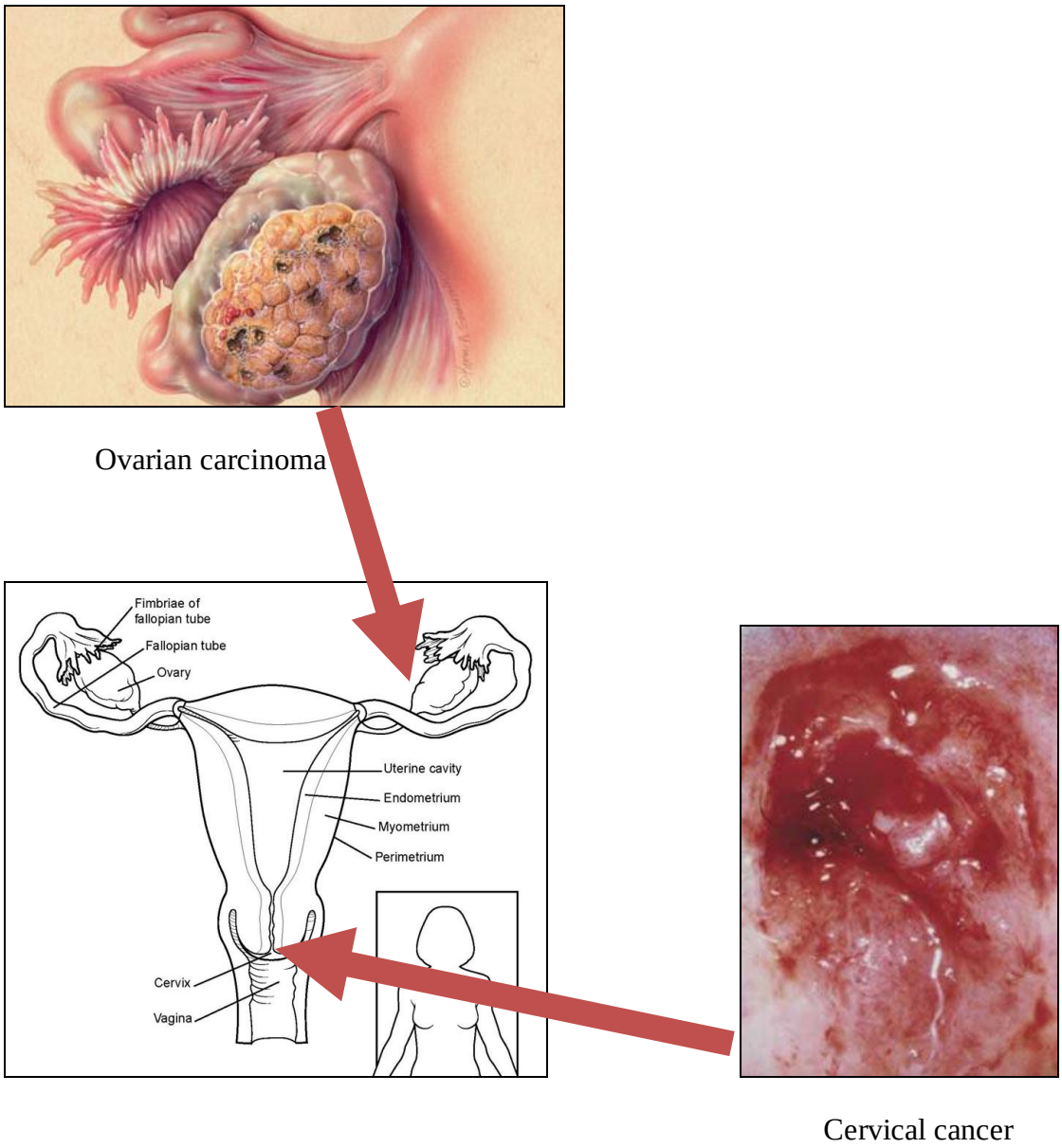


Figure 2: Ovarian carcinoma and cervical cancer

(Adapted with permission from http://www.medicinenet.com accessed on 1 February 2012)

1.2.1 Types of ovarian tumours and the risk factors

Ovarian tumours are named according to the type of cells the tumour develops from. Mainly there are three types of ovarian cancers which are the epithelial tumours, germ cell tumours and stromal tumours (Andrew & Jules, 2011). The epithelial ovarian tumours can be divided further into benign epithelial ovarian tumours, tumour of low malignant potential, malignant epithelial ovarian tumours and primary peritoneal carcinoma. The germ cell tumours can also be further categorised into teratoma, dysgerminoma, endodermal sinus tumour (yolk sac tumour) and choriocarcinoma (Andrew & Jules, 2011).

Up until now, the exact cause of ovarian cancer is still unknown. However, it is demonstrated that hereditary ovarian cancer generally occurs within one of two distinct genetic backgrounds. The first, hereditary breast and ovarian cancer (HBOC) syndrome, is attributable to germline mutations in the *BRCA1* or *BRCA2* tumour suppressor genes (Kuschel *et al.,* 2000; Lee *et al.,* 2000), while the second is associated with hereditary non-polyposis colorectal cancer (HNPCC), or Lynch Syndrome, which is attributable to a germline mutation in one of several genes located within the DNA mismatch repair pathway (Russo *et al.,* 2009; Lu *et al.*, 2008).

Recent evidence supports the notion that the genetic background underlying ovarian tumour genesis extends well beyond these familial conditions and that the development of fully malignant tumours involves the progressive acquisition of mutations in multiple genes, including *BRAF, KRAS, PTEN, Her2/neu, c-myc, p16,* and *p53* (Shih *et al.,* 1998; Pearson *et al*., 1998 Stanley *et al.,* 1995). Although these molecular alterations have been identified in a significant fraction of ovarian cancers, none of these mutations are diagnostic of malignancy or predictive of tumour behavior over time. Furthermore, the frequency of several of the above mutations appears to be highly dependent on the histological subtype of the tumour (Shih *et al.,* 1998).

The lifetime risk of developing ovarian cancer place at 1.39% and the risk factors for the development of ovarian cancer includes age of the individual if it is above forty (Ness *et al*., 1999), family history of ovarian, breast, cervical or colon cancer, early age of menarche, late menopause (Cramer *et al*., 2001) and nulliparity (Rodriguez *et al*., 2001). Other than age and genetic background, the risk factor associated with ovarian cancer includes the use of hormone replacement therapy or fertility drugs, diet and ethnicity (Folch and Soloane, 1957).

1.2.2 Ovarian cancer screening: current trend and obstacles

The failure to detect ovarian cancer at an early stage can cause high mortality to the ovarian cancer patients. Screening for early detection strategies are believed to have potential in improving patient survival (Baker *et al.,* 1994). At present, women who are identified as high risk of ovarian cancer need to rely on genetic counseling and screening of serum CA 125 and transvaginal sonography (Bast *et al.,* 2007). CA 125 is not a diagnostic or prognostic marker but its used have been demonstrated currently in monitoring treatment response and disease.

Although the detection and monitoring can be done in various ways such as CA 125, transvaginal sonography (TVS), doppler and morphological indices, each method is lacking of the specificity required in general population detection (MacDonald *et al.,* 1998). For example, the CA 125 assays showed only 50-60% sensitivity at stage I of the disease (Jacobs *et al.,* 1989). In addition, the CA 125 test also showed less sensitive to premenopausal women compared to post menopausal (Haaften-Day *et al.,* 2001).

A screening strategy that combines the use of tumour measured at specific intervals with ultrasound may represent a cost-effective strategy for early detection and may yield higher sensitivity and specificity (Jacob *et al.,* 2004; Menon *et al.,* 2001). However, this

strategy relies solely on CA 125 which unlikely provides a sufficient sensitivity for early stage diseases. Another strategy is to combine a potential biomarker with CA 125 in order to determine the risk stratification. Several papers had demonstrated the combination of CA 125 and Human Epididymal Protein 4 (HE4) had an improved sensitivity and specificity for earlier detection of ovarian cancer (Huhtinen *et al.,* 2009; Moore *et al.,* 2009; Moore *et al.,* 2008). A diagnostic analyser to detect HE4 is now commercially available in the market. However, HE4 is also reported to be elevated in all stages of endometrial cancer and its sensitivity is more towards early stage of endometrial cancer when compared to CA 125 (Moore *et al.,* 2008).

Since prevalence of ovarian cancer in general population is relatively low, any proposed strategy must demonstrate a minimum specificity of 99.6% and a sensitivity of more than 75% for early stage disease to achieve a positive predictive value of 10% and avoid an unacceptable level of false positive results (Jacob *et al.,* 2004; Menon *et al.,* 2001). Therefore, considering that the ovarian cancer is asymptomatic and the tumours can mainly develop from various types of the cells, there is an urgent need to develop additional informative biomarker, identification of a novel biomarker or combination of biomarkers that can detect small pre-symptomatic ovarian tumours and differentiate malignant from benign tumours with high level of sensitivity and specificity.

1.3 Cervical cancer

Cervical cancer is the second most common malignancy in women worldwide, and it remains as a leading cause of cancer-related death for women in developing countries. In Malaysia, cervical cancer is the second most common cancer that happens among women aged between 30-69 years (Othman, 2003). Cervical cancer mortality rates in Malaysia declined from 1985 to 1993 and then increased in 1997 (National Cancer Registry, 2002).

According to the latest third report (National Cancer Registry, 2008), a total of 4,057 cases of cervical cancer had been reported from 2003 until 2005. The incidence of cervical cancer in the Malaysian's Chinese is among the highest compared to the other races in the country and also the Chinese women in other Asian countries.

1.3.1 Human pappiloma virus and cervical carcinoma

The cervical cancer cells begin to grow in the normal cells lining of the cervix. Human Pappiloma Virus (HPV) is a DNA virus and high risk HPV has been accredited in 99.7% of invasive cervical cancer (Walboomers *et al.,* 1999). After infection, the cells of the cervix gradually develop pre-cancerous changes and then turn into cancer cells. However, only certain type of HPV are considered as high-risk types which include type 16, 18, 31, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 (Muñoz *et al.,* 2003).

Although the increasing scenario of cervical cancer is more likely due to the increasing of HPV infection (Adeeb *et al.,* 2007), the actual prevalence of HPV infection among Malaysian women is remain unknown since there is no large study on HPV in Malaysia. However, large studies on prevalence of HPV infection have been conducted in four Asian countries namely India, Vietnam, Korea and Thailand. The study showed that the age standardised prevalence for any HPV is 8.7%, with the high-risk types representing a large portion of the infected women (5.4%) (Clifford *et al.,* 2005).

Apart from HPV infection, other factors such as smoking, chlamydial infection, low diet intake of fruits and vegetables (Ghosh *et al.,* 2008), consumption of oral contraceptives and diethylstilbestrol (Hatch *et al.,* 2001) can cause cervical cancer although their contribution is small toward the disease.

1.3.2 Cervical cancer vaccine as a primary prevention

Since early year of 2009, media publicity on the prevention of cervical cancer has been made (http://health.asiaone.com/Health/Women) especially by taking human papilloma virus (HPV) vaccine also, known as cervical cancer vaccine (Ault *et al.*, 2007). In 2006, a vaccine called Gardasil was used to protect against HPV types 6, 11, 16 and 18 (The Future II Study Group in 2007). Another vaccine, Cervarix has been approved by the Food and Drug Administration in 2009 to be used in the United States to protect women against HPV types 16 and 18. Recommendation for HPV vaccination was also published on 2009 by the Federal Advisory Committee on Immunization Practices (ACIP).

Malaysian Ministry of Health had promoted and initiated the HPV vaccination to girls with the age of 13 since 2009. However, the issues of cost-effectiveness and long term benefits are yet to be answered. There are a few issues that have to be addressed such as the duration of protection, the need for booster and efficacy in older women. The efficacy of this vaccine program towards Malaysian secondary school students may reduce the risk factor of getting the cervical cancer. However, the statistical data revealing the involvement of pre-marital sexual intercourse since the age of 12 (Lee *et al.*, 2006) is somewhat disturbing. It is not impossible that the issue of when the vaccination among adolescents should begin might arise again in the future and the cost-effectiveness as well as efficacy of prevention might be questionable.

1.3.3 Cervical cancer screening program: current status

Detection of cervical cancer can be made if women undergo regular Papinacalau (PAP) smear test (Wright *et al.*, 2007). PAP smear is a medical procedure in which sample of cells from a woman's cervix covering the end of the uterus that extends into the vagina is collected and smeared on a microscope slide. Incidence and mortality due to cervical cancer

was significantly reduced in countries with organized PAP smear screening program (Laara *et al*., 1987).

According to the National Cervical Cancer Guidelines 2003 and Guidebook in PAP smear 2008, all sexuality active women age between 20-65 years shall undergo PAP smear screening annually for two consecutive years and if the PAP smear is normal in both occasions, the screening test can be continued once every three years. However, a community survey done by National Health and Morbidity Survey in 1996 found that the coverage of the PAP smear screening program was 26% among the Malaysian woman which indicates that awareness regarding PAP smear is relatively low.

Several possibilities was associated with what was stoping the Asian women from going to their regular yearly or three yearly PAP smears. Women were not empowered with the knowledge they need to seek preventive screening, especially older women since they are unlikely to visit family planning clinics (Anon, 1997). Beside that, strong belief in traditional medicine, feeling fear and discomfort towards the medical procedures are also the reasons why women put the PAP smear screening into denial (Cheah and Looi, 1999).

1.3.4 New technology for cervical cancer screening

An atypical squamous cell of undetermined significance (ASC-US) is a major limitation during the PAP smear test. New technologies for cervical cancer screening are currently developed and one example is by HPV testing using Hybrid Capture 2. The analysis was approved by the FDA in 2003 as a primary screening test. This is due to a study conducted on 7932 women with the median age of 34 years which had shown 100% sensitivity in detecting a histologically proven high grade squamous intraepithelial lesion (HGSIL) and higher specificity in women age more than 30 years (Clavel *et al.*, 2001).

Although the study involved a large number of individuals, strategies have to be streamlined to capture not only women age more than 30 years but also the reproductive age group from 20-30 years. In conjunction to the problems and the issues which is initially mentioned, an identification of a protein(s) as a novel molecular marker is such a favorable attempt in distinguishing healthy and cervical cancer women which not involved cells as a sample that might contribute to the insensitivity screening due to the age factor.

1.4 Cancer staging system

Cancer staging is a basic activity in the area of oncology. It is structured to represent a major prognostic factor in predicting patients' outcome and lending order to the complex dynamic behavior of a cancer (Benedet *et al.,* 2006). One of the major purposes of cancer staging is to offer a classification of a cancer's extent in order to provide a method of conveying one's clinical experience to other for the comparison of treatment methods without ambiguity.

Tumour classification is generally conceived so that the clinical and/or pathological spread is stratified into 4 stages: Stage I refers to a tumour strictly confined to the organ of the origin; Stage II describes disease that has extended locally beyond the site of origin to involve adjacent organs or structures; Stage III represents more extensive involvement such as wide infiltration reaching neighboring organs and Stage IV represents clearly distant metastatic disease (Pecorelli *et al.,* 2006). These four basic stages are then classified into sub stages, as a reflection of specific clinical, pathological, or biological prognostic factors within a given stage (Bösze *et al.,* 2001). Gospodarowicz *et al.,* in 1998 suggest that these certain factors such as the site of origin of the disease, its biology, and the extent of the disease at the time of presentation is required in order to optimally manage any malignant disease.

Over the last 30 years, all changes to the FIGO classification and staging system have been extensively discussed by the FIGO Committee on Gynecologic Oncology and put forward in agreement with and approved by the Union Against Cancer (UICC) tumour-node-metastasis classification (TNM) Committee, the American Joint Committee on Cancer (AJCC), and the World Health Organization. Tables 1 and 2 provide the current FIGO staging classifications published in the Twenty-sixth Volume of the FIGO Annual Report (Pecorelli *et al.,* 2006). Over the years, the UICC, AJCC, and FIGO have modified their staging systems for gynecological cancers so that all three systems are virtually identical (Gospodarowicz *et al.,* 1998).

Currently, an agreement between the three bodies ensures comparability of staging classifications for gynecologic malignancies and their representatives meet annually. The interaction among these bodies has led to the creation of uniform information shared within the scientific community (Pettersson *et al.,* 2001) thereby promoting continuous uniformity between all bodies. Further and joint efforts are constantly made to unify the FIGO and TNM classifications.

Table 1 Carcinoma of the ovary: FIGO nomenclature (Rio de Jeneiro 1988)

Stage I	Growth limited to the ovaries.
	Stage Ia: Growth limited to one ovary: no ascites present containing malignant cells. No tumour on the external surface; capsule intact.
	Stage Ib: Growth limited to both ovaries: no ascites present containing malignant cells. No tumour on the external surfaces; capsules intact.
	Stage Ic[a]: Tumour either Stage Ia or Ib, but with tumour on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
Stage II	Growth involving one or both ovaries with pelvic extension
	Stage IIa: Extension and/or metastases to the uterus and/or tubes.
	Stage IIb: Extension to other pelvic tissues.
	Stage IIc[a]: Tumour either Stage IIa or IIb, but with tumour on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washing.
Stage III	Tumour involving one or both ovaries with histologically-confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or ingual nodes. Superficial liver metastases equal Stage III. Tumour is limited to the true pelvis, but with histologically-proven malignant extension to small bowel or omentum.
	Stage IIIa: Tumour grossly limited to the true pelvis, with negative nodes, but with histologically-confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentry.
	Stage IIIb: Tumour of one or both ovaries with histologically-confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter: nodes are negative.
	Stage IIIc: Peritoneal metastasis beyond the pelvis >2cm in diameter and/or positive retroperitoneal or ingual nodes.
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV.

[a] In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage Ic or IIc, it would be a value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected peritoneal washings, or ascites.

Reprinted from: Heintz APM, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, *et al*. Carcinoma of the ovary. Int J Gynecol Obstet 2006; 95 (Suppl 1):S163.

Table 2: Carcinoma of the cervix uteri: FIGO nomenclature (Montreal, 1994)

<table>
<tr><td colspan="2">Stage 0
Carcinoma in situ, cervical intraepithelial neoplasis Grade III.</td></tr>
<tr><td rowspan="2">Stage I
The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded).

Stage 1a: Invasive carcinoma which can be diagnosed only by microscopy. All macroscopically visible lesions – even with superficial invasion – are allotted to Stage Ib carcinomas. Invasion is limited to a measured stromal invasion with a maximal depth of 5.0 mm and a horizontal extension of not >7.00 mm. Depth of invasion should not be >5.0 mm taken from the base of the epithelium of the original tissue should not change the stage allotment.</td><td>Stage Ia1: Measured stromal invasion of not >3.0 mm in depth and extension of not >7.0 mm.</td></tr>
<tr><td>Stage Ia2: Measured stromal invasion of >3.0 mm and not >5.0 mm with an extension of not >7.0 mm.</td></tr>
<tr><td rowspan="2">Stage Ib: Clinically visible lesions limited to the cervix uteri or preclinical cancers greater than Stage Ia</td><td>Stage Ib1: Clinically visible lesions not >4.0 cm.</td></tr>
<tr><td>Stage Ib2: Clinically visible lesions >4.0 cm.</td></tr>
<tr><td rowspan="2">Stage II
Cervical carcinoma invades beyond uterus, but not to the pelvic wall or to the lower third of vagina.</td><td>Stage IIa: No obvious parametrial involvement</td></tr>
<tr><td>Stage IIb: Obvious parametrial involvement.</td></tr>
<tr><td rowspan="2">Stage III
The carcinoma has extended to the pelvic wall. On rectal examination, there is no cancer-free space between the tumour and the pelvic wall. The tumour involves the lower third of the vagina. All cases with hydronephrosis or nonfunctioning kidney are included, unless they are known to be due to other cause.</td><td>Stage IIIa: Tumour involves lower third of the vagina, with no extension to the pelvic wall.</td></tr>
<tr><td>Stage IIIb: Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney.</td></tr>
<tr><td rowspan="2">Stage IV
The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to Stage IV.</td><td>Stage IVa: Spread of the growth to adjacent organs.</td></tr>
<tr><td>Stage IVb: Spread to distant organs like the lungs.</td></tr>
</table>

Reprinted from: Quinn MA, Benedet JL, Odicino F, Maisonneuve P, Beller U, Creasman WT, *et al.* Carcinoma of the cervix uteri. Int J Gynecol Obstet 2006;95 (Suppl 1):S43.

1.5 Urinary proteins

Urine has been used over the centuries mainly for the study and monitoring of renal physiology and pathology (Papale *et al*., 2007). For example, the presence of albumin in the urine has been measured as an indicator for renal disease whereas human chronic gonadotropin which is present in the urine is used in the pregnancy test kit to detect pregnancy of women. Recently, urine was extensively studied as a potential source of biomarkers due to the non-invasive nature of getting the sample from the patient (Pieper *et al*., 2004; Spahr *et al*., 2001). Approximately up to 150 mg of proteins and peptides are excreted in urine per day from individual who does not have any kidney problem.

The proteins from the urine were usually originated from glomerular filtration of blood plasma, cell apoptosis (Pavenstadt *et al.,* 2003), secretion of exosomes by epithelial cells and proteolytic cleavage of cell surface glycosylphosphatidylinositol-linked proteins (GPI). Around 50% of these proteins are derived from glomerular filtration (Zhou *et al*., 2006). Low molecular weight proteins which are less than 10 kDa can pass freely through glomerular barriers and almost none of high molecular weight proteins or only a fraction of proteins with middle molecular weight reach renal tubules (Christensen and Birn, 2001). However, proteins that are abundant in the blood plasma such as albumin and various globulins can pass through the glomerular filter in substantial amounts.

1.5.1 Application of urine in diseased detection

A change in a given soluble protein concentration in the blood plasma, or a change in the function of the glomerular filter or an alteration in the proximal tubule scavenging system can result to a change in its amount in the final urine. Changes in the urinary proteome may therefore be used to detect not only abnormalities within the kidney and the

urogenital tract but also systemic disease associated with small circulating protein and peptide markers that can pass through the glomerular filter (Jonathan and Topham, 2007).

In the search for new protein biomarker candidates with clinical diagnostic value, substantial progress was made in the proteomic analysis of serum samples of patients with different cancers (Pang *et al.,* 2010; Cordero *et al.,* 2008; Maurya *et al.,* 2007). In contrast, fewer studies have been carried out on the urine samples of cancer patients. However, the identification of more than 1500 proteins in the urine of healthy donors which had being performed using advanced mass spectrometry techniques (Adachi *et al*., 2006) gave an advantage to the new researches in term of streaming down the analytical strategy and comparison purposes. Investigation using the SELDI-TOF-MS technique done on urine of patients with ovarian carcinoma which is restricted to the low molecular weight peptide analysis (Ye *et al.,* 2006) proves that wider coverage could be done in order not to miss out any potential biomarker. Thus, making a proteomic as a favorite approach among the researchers to understand diseases and this perhaps could lead to the development of more effective treatments.

1.5.1.1 Proteomic and urinary tract disease

Interstitial cystitis (IC), an example of urinary track disease, is recently being studied using proteomic technique. IC is a chronic disease that consists of urinary urgency, frequency and bladder pain (Curhan *et al.,* 1999). The urge of the study is basically due to often delay in the treatment of IC since there is no known cause or reliable method of diagnosis. Investigation using proteomic approached manage to identify a candidate biomarker for the diagnosis of IC where the increased of anti proliferative factor (APF) activity in the bladders of patients with IC were demonstrated (Zhang *et al.,* 2001). Another attempt to understand the disease was done by correlating the patient quality-of-life scores

and IC symptom scores in order to determine whether there is a linear association between quantitative biomarker analysis and standardised measures of activities of daily living, symptom severity, and pain (Canter *et al.,* 2008). Although this study had declared the exclusion of APF protein in their study, they had shown a promising result by demonstrating the correlation between IC severity with the decrease in Tamm-Horsfall protein (THP) and kininogen in the urine of the patients.

1.5.1.2 Proteomic and cancer

Colorectal cancer is the second most common cause of cancer related to death in a developed country but early diagnosis often leads to a complete cure (Burt *et al.,* 2010). Many cases of colon cancer have no symptom; however the blood in the stool (Lieberman *et al.,* 2009) and abdominal pain and tenderness in the lower abdomen (Millham *et al.,* 2010) may indicate colon cancer. Foecal occult blood test (FOBT) is used to screen the disease and showed a significant reduction in mortality (Hewitson *et al.,* 2007). It is a test to check stool for blood that can only be seen with a microscope. However, this test cannot tell whether the blood is from colon or from other parts of the digestive tract such as stomach. If this test is positive, a procedure to look inside the rectum and colon for polyps, abnormal areas called colonoscopy is needed to find the cause of bleeding.

Another stool-based approach is stool DNA test (sDNA). Instead of looking for blood in the stool, this test looks for abnormal DNA from cancer or polyp cells. Although the test appears effective and cost-effective, but the test is poorer compared to other strategies such as FOBT and colonoscopy (Song *et al.,* 2004). A protein biomarker namely carcinoembryonic antigen (CEA), is a tumour marker used to trace cancer in gastrointestinal tract. However, it is not an effective screening test due to the low sensitivity in patients at early stage of the disease (Hurst *et al.,* 2007).

Up until now, no blood or stool biomarkers with both high sensitivity and specificity for potentially curable early stage disease have been validated for clinical use. A proteomic approaches have shown that in principle, they are capable of uncovering biomarkers with high sensitivity and specificity. For example, Ward *et al.,* (2006) manage to find a panel of biomarker for colon cancer with greater sensitivity and specificity when compared to CEA In their study, although there is a considerable overlapping between different approach of proteomic technique such as SELDI and MALDI data, all the profiling method detect unique peaks with high sensitivity. Ward *et al.,* (2008) also successfully identified hepcidin-20, β2-microglobulin and 18 residue fragment of the α-subunit fibrinogen in the urine which could be associated with colorectal cancer.

1.5.1.3 Proteomic and animal disease

Recent studies also demonstrate that the use of urine for the identification of disease-induced biomarkers is not only being applied in human urinary proteins but also in animal. Bovine Spongiform Encephalopathy (BSE) and other Transmissible Encephalopathy (TSE) diseases are uniformly fatal degenerative syndromes of the central nervous system (CNS) that occurred in cattle (Smith *et al.,* 2003). This disease is untreatable. A new human variant of Creutzfeldt-Jakob Disease (vCJD) is thought to have been caused by dietary exposure to BSE infected cattle, thus led to profound changes in the production and trade of the agricultural goods (Peden *et al.,* 2004).

The rapid test currently approved for BSE monitoring in slaughtered cattle are all based on the detection of the disease related isoform of the prion protein, $PrP^{d,}$ in brain tissue and therefore are only suitable for post-mortem diagnosis. In order to assess the health of breeding stock for export purposes where post-mortem testing is not an option,

there is a demand for ante-mortem test based on matrix or body fluid that would permit easy access and repeated sampling.

A study of 2-DE and mass spectrometry analyses were used to identify proteins exhibiting differential abundance in the urine of BSE infected cattle and age matched controls of the disease (Sharon *et al*., 2008). This study suggested that in principle, it is possible to identify biomarkers in urine. Moreover, the biomarkers found as immunoglobulin gamma-2 chain C region, cystatin, cathelicidin, uroguanylin and clusterin in this study are useful in the diagnosis, prognosis and monitoring of disease progression of transmissible spongiform encephalopathy diseases.

In conclusion, to date, proteomic experiments that have been conducted on urine were not confined to patients suffering from diseases of the genitourinary system (Buhimschi *et al.,* 2004) but also others such as mentioned above, it has been carried out on those with atherosclerosis (Von-Zur-Muhlen *et al.,* 2009), sleep disorder (Gozal *et. al.,* 2009), cancers of the bladder (Kreunin *et al.,* 2007), pancreas (Weeks *et al.,* 2008; Kojima *et al.,* 2008) and lung (Tantipaiboonwong *et al.,* 2005). Therefore, it is not impossible to find a biomarker in urine of the patients with diseases such as ovarian carcinoma and cervical cancer too.

1.5.2 Proteomic methods used in this study

In this present study, 2-DE coupled with mass spectrometry identification and SELDI-TOF were adopted for the screening of diseased and control individuals. Due to the different approach on protein detection, these two techniques complement each other where wider range of proteins could be screened for potential biomarker.

1.5.2.1 Two dimensional gel electrophoresis coupled with MALDI-TOF-MS

The 2-DE method was preferred in this study due to the high resolution it gives. More importantly, individual protein spots could be visualized, which was not able to be done by other proteomic approach. It is also applicable in the studies requiring the quantitative analysis in comparing the proteome between different samples (Stein & Zvelebil, 2002). This technique separate complex protein mixtures based on two discrete steps. The first-dimension step is the isoelectric focusing (IEF) which separates proteins according to their isoelectric points (pI) and the second dimension step is the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which separates proteins according to their molecular weight. To identify the resolved protein in 2-DE gel, the protein spots can be excised, digested and subjected to mass spectrometry.

Mass spectrometry is an analytical technique that measures the mass of molecules based upon the motion of a charged particle in an electric or magnetic field. The data obtained are then exported in a format to the database search program, Mascot (Matrix Science Ltd, London, UK). The data was searched against 'All entries' in the SwissProt database. High scores in the database search indicated a likely match and confirmed by operator inspection.

1.5.2.2 Surface enhanced laser desorption/ionization time of flight-mass spectrometry (SELDI-TOF-MS)

Unlike 2-DE where the protein could be visualised and identified, SELDI-TOF will only gives the profile of protein samples based on the target chip used. Comparison between samples still can be done based on the profile obtained. SELDI-TOF technique is preferred because of its reproducibility and its capacity to permit rapid comprehensive large-scale analysis of individual proteins within complex protein mixtures. With the

reduction in sample complexity, there is an improved ability to detect lower abundance proteins (Lin *et al.*, 2004). SELDI-TOF allows protein profiling from a variety of complex biological materials such as serum, blood, plasma, tissue, urine, saliva, cell lysis products with limited sample preparation. Complex sample analysis, however, involved sophisticated approaches including digestion and labelling of the target proteins (Ferguson et al., 2003). In contrast, the label-free quantification of native proteins is an inherent part of the SELDI-TOF process and does not require any additional preparation or labelling.

In the SELDI-TOF method, protein solutions are applied to the spots of ProteinChip arrays, which have been derivative with planar chromatographic chemistries. The proteins actively interact with the chromatographic array surface, and become sequestered according to their surface interaction potential as well as separated from salts and other sample contaminants by subsequent on-spot washing with appropriate buffer solutions. Furthermore, protein interaction studies or enzymatic reactions may be carried out directly on-spot under physiological conditions. The chromatographic surfaces provide a very good support for the crystallization of matrix and target proteins, resulting in the formation of a homogenous layer on the spot, thereby delivering an ideal crystalline surface for the subsequent analysis.

There are several protein chips array available in the market with different purpose of analysis. The protein chips are such as CM10, a weak cation exchange chip that capture molecules that have positive surface charges; Q10, a strong anion exchange chip that capture molecules that have negative surface charges; IMAC 30, an immobilised metal affinity that capture molecules which bind polyvalent metal ions such as nickel, copper, zinc, iron and gallium; H50, a hydrophobic protein chip array that capture large proteins through hydrophobic or reverse phase interaction; and NP20, a normal phase protein chip

that also known as a general protein binding surface. It is recommended for hydrophilic proteins.

The spectrums obtained from the SELDI-TOF are further analysed with ChipergenExpress Data Manager and Biomarker Pattern Software™ (BPS). ChiphergenExpress Data Manager is an application within ProteinChip Biomarker System used to generate peak cluster and export these clusters into a file for analysis in Biomarker Patterns™ Software (BPS). BPS is a single procedure that can be used to analyse either categorical (classification) or continuous (regression) data with the implementation of CART (**C**lassification **a**nd **R**egression **T**rees). A defining feature of CART is that it presents its results in the form of decision trees. This decision trees will gives potential protein peaks that can be determined as a potential biomarker/s.

1.6 Aim of the investigation

The aim of this study was to screen urine from patients with ovarian cancer and cervical cancer for a potential biomarker(s). This was done by achieving the following objectives:

a. To develop 2-DE profile of urinary proteins from control and patients with ovarian carcinoma and cervical patients.
b. To compare 2-DE profile of urinary proteins from control and patients with ovarian carcinoma and cervical patients using Image Master 2D Paltinum software.
c. To identify aberrantly expressed protein using MALDI-TOF-MS/MS.
d. To verify the identified protein using Western Blotting technique.
e. To screen the low molecular weight proteins using SELDI-TOF-MS.
f. To analyse spectrum obtained from SELDI-TOF-MS with ChipergenExpress Data Manager and Biomarker Pattern Software™ (BPS) for potential biomarker(s).

Chapter 2
MATERIALS AND METHODS

SECTION A: MATERIALS

2.1 Urine collections

Urine samples were collected from patients which were newly confirmed with stages II and III ovarian carcinoma (n = 11) and cervical cancer (n = 11), prior to chemotherapy treatment, at the University of Malaya Medical Centre (UMMC), Kuala Lumpur. Control urine samples were collected randomly from age-matched cancer negative women (n = 15). Samples obtained were with consent and approval granted by the ethical committee of UMMC in accordance to the ICH GCP guideline and the Declaration of Helsinki. The subjects were of different ethnic background (Malay, Chinese and Indian).

2.2 General material

The materials used in this study and their respective suppliers are listed below.

2.2.1 Chemicals and reagents

All chemicals were both analytical grade and proteomic grade and were purchased from Sigma Aldrich Company, St. Louis, United States of America (USA) with the exception of the following:

a) *Amersham Pharmacia Biotech, Uppsala, Sweden*

   TEMED (N, N, N',N'-Tetrametyhlenediamine)

b) *Merck, Darmstadt, Germany*

   Glycine and Urea

c) *Bio-Rad, Hercules, USA*

   Bis N, N',-methylene-bis-acrylamide and Sodium dodecyl sulphate (SDS)

d) *Bio-Rad Laboratories, Richmond, USA*

   Ammonium persulphate

e) *Merck, Darmstadt, Germany*

Acetic acid, Ammonium sulphate, Sodium chloride

f) *Pierce Biotechnology, Rockford, USA*

Pierce® BCA Protein Assay Kit

g) *Thermo Scientific Pierce Protein Research Products, Rockford, USA*

SnakeSkin Dialysis Tubing, 10K MWCO

h) *Promega, Wisconsin, USA*

Trypsin Gold

2.2.2 Two-Dimensional gel electrophoresis

IEF immobiline dry strips (pH 3-10), pharmalyte 3-10 and drystrip cover fluid were supplied by GE Healthcare, Uppsala, Sweden.

2.2.3 Antibodies

a) *Abcam, Cambridge, United Kingdom*

Anti-human CD59

b) *Abnova, Jhongli, Taiwan*

Anti-human kininogen-1

c) *Sigma Aldrich Company, St. Louis, USA*

Anti-albumin, Anti-rabbit IgG (whole molecule)-Peroxidase, Anti-mouse IgG (Fc Specific)-Peroxidase

SECTION B: METHODS

2.3 Urine samples

2.3.1 Pre-treatment of urine samples

Sodium azide was immediately added to the urine upon collection to a final concentration of 20 mM. The samples were centrifuged at 10,000 rpm at 4^{o}C and the supernatant was collected and dialysed using 10-kDa cutoff dialysis tubing (Pierce, Rockford, USA) against distilled water. The urine proteins were aliquot, freeze-dried and kept at –20ºC. Protein content was determined using the Pierce BCA protein assay kit.

2.3.2 Determination of urine sample concentration

The protein concentration of urine samples was determined using the BCA (Biochichoninic acid) protein assay where the amount of protein in the urine sample was estimated from a constructed standard curve. This is important in order to standardize the amount of protein to be analyzed with two-dimensional gel electrophoresis and SELDI-TOF-MS.

2.3.2.1 Preparation of the BCA Working Reagent (WR)

The following formula was used to determine the total volume of WR required:

(# standards + # samples) × (# replicates) × (volume of WR per sample) = total volume WR required.

The WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). When Reagent B was first added to Reagent A, turbidity was observed which quickly disappeared upon mixing to yield a clear, green WR. The WR was stored at room temperature (RT) and stable for several days when stored in a closed container. Nine vials labeled with A to I was prepared to perform different protein

concentration. To prepare the standard curve, the stock with the concentration of 2000 µg/ml BSA was diluted as follow:

Dilution Scheme for Standard Microplate Procedure (Working Range = 20-2000 µg/ml)			
Vial	Volume of Diluent (µl)	Volume and Source of BSA	Final BSA Concentration (µg/ml)
A	0	300 µl of stock	2000
B	125	375 µl of stock	1500
C	325	325 µl of stock	1000
D	175	175 µl of vial B dilution	750
E	325	325 µl of vial C dilution	500
F	325	325 µl of vial E dilution	250
G	325	325 µl of vial F dilution	125
H	400	100 µl of vial G dilution	25
I	400	0	0 (Blank)

2.3.2.2 Protein concentration measurement by microplate procedure

The pellete of freeze dried urine sample was redissolved with 1 ml deionized water. Twenty five µl of each standard and urine samples were pipetted into a microplate well (working range = 20-2000 µg/ml). Two hundred µl of the WR was added to each well and the plate was shaked thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37ºC for 30 minutes. The plate was cooled down to RT and the absorbance was measured at 562 nm on a plate reader. A graph of absorbance against protein concentration was plotted and the concentration of urinary protein was determined based on graph (Figure 3).

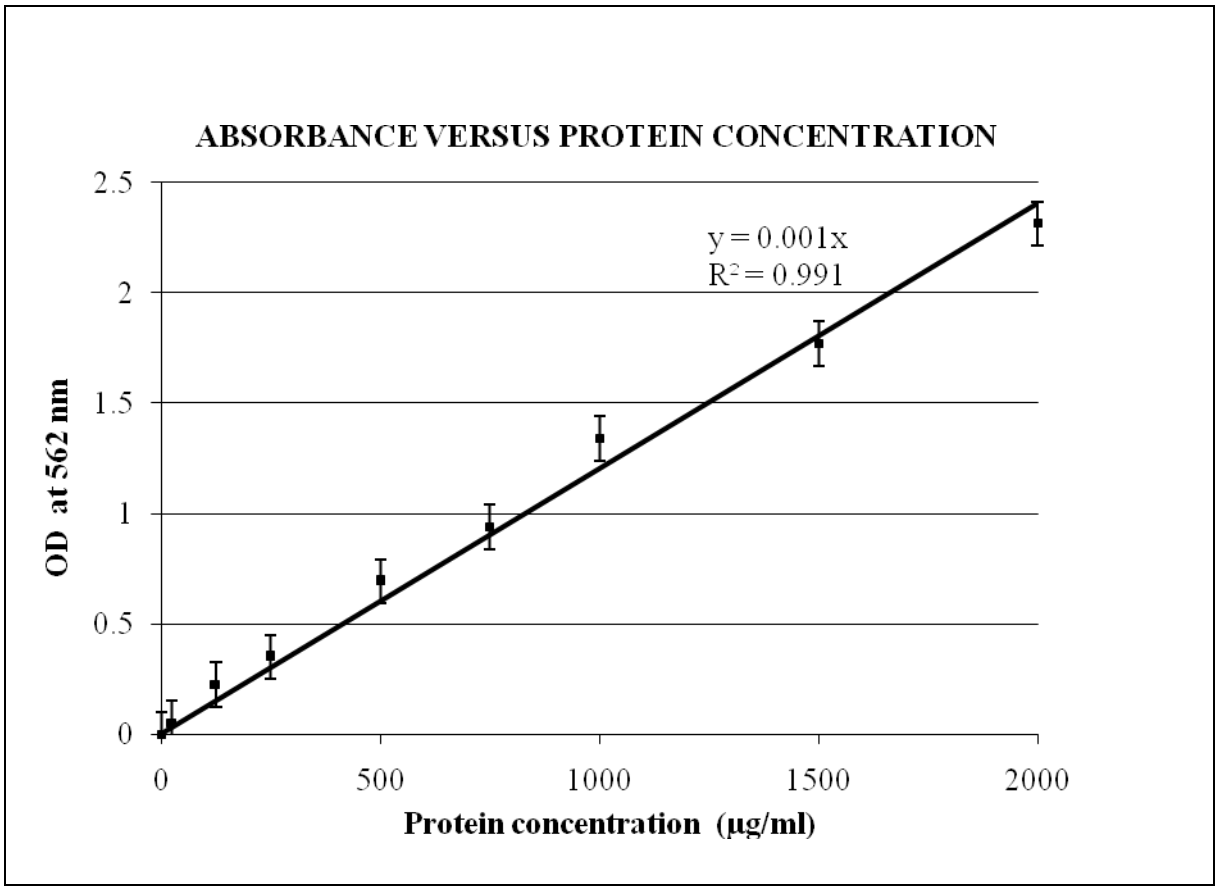


Figure 3: Standard curve of protein concentration

The standard curve was constructed based on BSA solution with concentrations ranging from 20-2000 µg/ml using Pierce BCA protein assay kit. The absorbance was read at 562 nm. Protein concentration in the urine samples was estimated based on the standard curve.

2.4 Two-dimensional gel electrophoresis (2DE)

2.4.1 First dimension electrophoresis

2.4.1.1 Stock solution

Rehydration solution with pharmalyte [8 M urea, 0.5% (v/v) Pharmalyte 3-10, 0.5% (v/v) NP-40]

Urea	12.00 g
Pharmalyte 3-10	0.50 ml
NP-40	0.13 ml
A few grains of Orange G	

The solution was made up to 25 ml by addition of deionized water and was stored at -20ºC until used. Prior to use, 7 mg of DTT was added to every 2.5 ml of rehydration solution.

2.4.1.2 Rehydration of immobiline dry strip

First, 300 µg of freeze-dried of urinary protein was dissolved in 200 µl rehydration solution. The sample was centrifuged at 5000 rpm for 15 minutes before it was pipetted into the reservoir slot of the immobiline drystrip reswelling tray (GE Healthcare, Uppsala, Sweden) which had been leveled by turning its leveling feet. After loading the sample mixture into the reswelling tray, 11 cm immobilized pH gradient (IPG) drystrips with the pH gradient of 3-10 L (linear) was positioned with the gel side down onto the solution. Subsequently, IPG drystrip cover fluid was pipetted onto the IPG strip until the entire strip was covered to minimize evaporation and to prevent urea crystallization. The strip was allowed to rehydrate for 18 hours at room temperature to ensure complete uptake of sample by IPG strip.

2.4.1.3 First dimension run

The temperature of the Multiphor™ II Electrophoresis Unit (GE Healthcare, Uppsala, Sweden) was set at 20ºC using a thermostatic circulator Eyela CA-1310 (Tokyo Rikakikai Co., Tokyo, Japan). Nine ml of dry strip cover fluid was pipetted onto the center of the cooling plate. The immobilline drystrip tray was positioned on the cooling plate and 9 ml of dry strip cover fluid was poured on the tray. Then, the drystrip aligner was placed inside the tray, on top of the dry strip cover fluid. IPG strips were positioned inside the grooves of the aligner with the acidic end facing the top of the tray. Moistened electrode strips were placed across the cathodic and anodic ends of the aligned IPG strips. Next, each IPG strip was overlaid with dry strip cover fluid to ensure good thermal contact. The electrodes in the Immobiline Dry Strip tray were ensured to be connected before placing the lid to the EPS 3501 XL (GE Healthcare, Uppsala, Sweden) power supply. The first dimension isoelectric focusing running conditions was as follow:

Phase	**Voltage (V)**	**Miliampere (mA)**	**Watt (W)**	**Volt-hour (Vh)**
1	300	2	5	1
2	3500	2	5	2900
3	3500	2	5	9100

Once the first dimension electrophoresis was completed, the focused strips were either used immediately for the second-dimension separation or were kept individually in screw-cap tubes at -80ºC. The strips that have been kept were used within 2 weeks.

2.4.2 Second-Dimension Electrophoresis

2.4.2.1 Stock solutions

Monomer stock solution (Solution A) [30% acrylamide, 0.8% N,N'-methylenebisacrylamide]

Acrylamide	60.0 g
N,N'-methylenebisacrylamide	1.6 g

The solution was made up to 200 ml with MiliQ water. It was filtered and kept in a dark bottle.

4× Resolving gel buffer (Solution B) [1.5 M Tris-HCl, pH 8.8]

Tris base	181.7 g

The Tris base was dissolved with 750 ml MiliQ water. After adjusting the pH to 8.8 with HCl, the solution was made up to 1 L with MiliQ water.

10% SDS (Solution C)

SDS	5 g

The solution was made up to 50 ml with MiliQ water.

10% Ammonium persulfate (Solution D)

Ammonium persulfate	0.1 g

The solution was made up to 1 ml with MiliQ water and prepared just prior to use.

SDS equilibration buffer [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS]

1.5 M Tris-HCl, pH 8.8	10.00 ml
87% (v/v)Glycerol	69.00 ml
Urea	72.07 g
SDS	4.00 g

The solution was made up to 200 ml with deionized water. The solution was stored at -20˚C.

5% Agarose sealing solution

Agarose	0.50 g
SDS electrophoresis buffer	100.00 ml

The mixture was swirled to disperse and heated in a microwave oven until the agarose was completely dissolved.

SDS electrophoresis buffer [25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS]

Tris-base	15.15 g
Glycine	72.00 g
SDS	5.00 g

The solution was made up to 5 L with deionized water.

2.4.2.2 Preparation of 12.5% homogenous SDS-PAGE gel

The Hoefer SE 600 (GE Healthcare, Uppsala, Sweden) was used for second-dimension electrophoresis. This electrophoresis unit consists of gel casting stand and glass plates to be assembled like a club sandwich. Four gels were run at one time using the club

sandwich divider. A vertical 12.5% gel was prepared by mixing the stock solution according to the following recipe:

Stock Solution	**Volume of stock solution**
Solution A (ml)	41.70
Solution B (ml)	25.00
Solution C (ml)	1.00
Top up with double distilled water (ml)	31.80
*Solution D (µl)	500.00
*TEMED (µl)	33.00
Total volume (ml)	100.00

*TEMED need to be added immediately once solution D was added into the solution.

After mixing the solutions, the gel solution was poured slowly between the gaps of sandwich-assembled glass plates. It was then overlaid with distilled water and was allowed to polymerize for a minimum of 1 hour.

2.4.2.3 Equilibration of IPG strips

Equilibration of IPG strip was performed in two discrete steps. First, the strip was equilibrated with 5 ml SDS equilibration buffer containing 50 mg DTT for 15 minutes with gentle shake on a shaker. The solution was then replaced with the SDS equilibration buffer containing 4.5% (w/v) iodoacetamide and a few grains of Bromophenol Blue. The strip was then shaken for another 15 minutes. The IPG strip was removed from the tube and rinsed

with SDS electrophoresis buffer before it was placed on the casted 12.5% homogenous SDS-PAGE gel. The strip was sealed with 0.5% agarose sealing solution.

2.4.2.4 Second-dimension run

The temperature of the SDS electrophoresis buffer in the 2-DE tank (Hoefer SE 600, Uppsala, Sweden) was maintained at 16ºC using a thermostatic circulator. Electrophoresis was performed at a constant current in two steps as follow:

Phase	Voltage (V)	Milliampere (mA)	Watt (W)	Time (Hour)
1	50	25	1.4	0.5
2	600	25	15	2

The stated values for miliampere and watt were per gel. Electrophoresis was stopped once the dye front was approximately 1 cm from the bottom of the gel. Then, the gel was removed from its cassette and the protein spots were visualized by silver staining.

2.5 Silver staining

The silver staining method was conducted according to the method introduced by Heukeshoven and Dernick (1988), with some modifications. A hundred and twenty five ml of solution was used per gel.

2.5.1 Stock solutions

Fixing solution [40% (v/v) ethanol and 10% (v/v) acetic acid]

Methanol	400.00 ml
Acetic acid	100.00 ml

The solution was made up to 1 L with double distilled water.

Sentizing solution [30% (v/v) ethanol, 0.13% (v/v) glutaryldehyde, 0.5 M sodium acetate, 8 mM sodium thiosulphate]

Ethanol	75.00 ml
50% (w/v) glutaryldehyde	0.75 ml
Sodium acetate trihydrate	17.00 g
Sodium thiosulphate pentahydrate	0.50 g

The solution was made up to 250 ml with double distilled water.

Silver solution [5.9 mM silver nitrate, 0.02% (v/v) formaldehyde]

Silver nitrate	0.25 g
35% (v/v) Formaldehyde	50.00 μl*

The solution was made up to 250 ml with double distilled water.

Developing solution [0.24 M sodium carbonate, 0.02% (v/v) formaldehyde]

Sodium carbonate	6.25 g
35% (v/v) Formaldehyde	50.00 μl*

The solution was made up to 250 ml with double distilled water.

Stop solution [40 mM EDTA-$Na_2.2H_2O$]

EDTA-$Na_2.2H_2O$	3.65 g

The solution was made up to 250 ml with double distilled water.

Preserving solution [10% (v/v) glycerol]

Glycerol	25.00 ml

The solution was made up to 250 ml with double distilled water.

(*) All these components were added immediately before use.

2.5.2 Silver staining protocol

All incubation steps of silver staining were carried out with gentle shaking on a shaker. After completion of the electrophoresis run, the gel was immersed immediately in the fixing solution and left for at least 30 minutes to allow SDS to diffuse out and to precipitate the proteins. Fixing solution was then replaced with the sensitising solution and the gel was incubated overnight. After that, the gel was washed three times with distilled water for 5 minutes. Then, the gel was incubated in silver solution for 40 minutes. The gel was then rinsed with approximately 50 ml of developing solution and left to develop in fresh developing solution. Once protein spots were developed on the gel, the developing solution was removed and the colour development was stopped by adding stop solution. The gel was left in the stop solution and shaken for 10 minutes. The gel was washed again with distilled water for 5 minutes with one change of water.

2.5.3 Gel Scanning and image analysis

The silver stained gel was scanned using Image Scanner III (GE Healthcare, Uppsala, Sweden). Urinary protein profiles of healthy control women and patients with ovarian cancer and cervical cancer were analyzed for abnormally expressed proteins using Image Master 2D Platinum Software 7.0 (GE Healthcare, Uppsala, Sweden). The levels of proteins in each sample were calculated as a percentage of volume contribution (% vol). In order to eliminate the possible variations due to staining, the volume of contribution refers

to the volume percentage of a protein taken against the total spot volume of all the proteins. The Student's t-test was used to analyze the significance of difference between control and each disease.

2.6 MALDI-TOF mass spectrometry analysis for protein identification

To identify the proteins of interest that were differentially expressed, the spots were subjected to MALDI-TOF mass spectrometry for protein identification. Proteins separated by gel electrophoresis can be visualized using a number of staining methods, however only some of these are compatible with protein digestion and mass spectrometric analysis. Therefore, preparation of prep-gel was required and the gel need to be stained with vorum silver stained according to the method of Shevchenko *et al.* (1996) for the protein identification analysis of abnormally expressed urine protein found in ovarian and cervical cancer patients.

2.6.1 Vorum silver staining

Two hundred ml of solution was used per gel. This staining method was different from method described previously in Section 2.5 mainly due to omittion of glutaryldehyde.

2.6.1.1 Stock solutions

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid and 0.048% (v/v) formaldehyde]

Methanol	200.00 ml
Acetic acid	48.00 ml
35% (v/v) Formaldehyde	190.00 µl

The solution was made up to 400 ml with MiliQ water.

Wash solution [35% ethanol]

Ethanol	420.00 ml

The solution was made up to 1.2 L with MiliQ water.

Sensitizing solution [1 mM sodium thiosulphate]

Sodium thiosulphate	0.10 g

The solution was made up to 400 ml with MiliQ water.

Silver solution [0.02% (w/v) silver nitrate, 0.076% (v/v) formaldehyde]

Silver nitrate	0.80 g
35% (v/v) Formaldehyde	288.00 µl

The solution was made up to 400 ml MiliQ water.

Developing solution [6% (w/v) sodium carbonate, 0.0004% (v/v) sodium thiosulphate, 0.05 % (v/v) formaldehyde]

Sodium carbonate	48.00 g
Sensitizing solution	16.00 ml
35% (v/v) Formaldehyde	378.00 µl

The solution was made up to 800 ml with MiliQ water.

Stop solution [50% (v/v) methanol, 12% (v/v) acetic acid]

Methanol	200.00 ml
Acetic acid	48.00 ml

The solution was made up to 400 ml with MiliQ water.

Preserving solution [1% (v/v) acetic acid]

Acetic acid	10.00 ml

The solution was made up to 1 L with MiliQ water.

2.6.1.2 Vorum Silver Staining protocol

All incubation steps of silver staining were carried out with gentle shaking on a shaker. After completion of the electrophoresis run, the gel was immersed immediately in the fixing solution and left for at least 2 hours to allow SDS to diffuse out and to precipitate the proteins. Following that, the gel was washed three times with washing solution for 20 minutes. Washing solution was then replaced with the sensitizing solution and the gel was incubated for 2 to 3 minutes. The gel was then rinsed three times with MiliQ water for 5 minutes. Then, the gel was soaked in silver solution for 20 minutes and again rinsed three times with MiliQ water for 5 minutes. The gel was then soaked in developing solution and left to develop within 3 to 5 minutes. Once protein spots or bands were developed on the gel, developing solution was removed and the colour development was stopped by adding stop solution. The gel was left in the stop solution and shaken for 5 minutes. The gel was kept in preserving solution and kept in 4ºC until the spots were cut out for MALDI-TOF analysis.

2.6.2 MALDI-TOF analysis

Protein identification was performed by mass spectrometry using 4800 Plus MALDI TOF/TOF™ Analyser (Applied Biosystem, Concord, Canada). Protein spots of interest were excised from the silver stained gels and subjected to in-gel digestion according to the method of Shevchenko *et al.* (1996).

2.6.2.1 Stock solutions

Destaining solution [15 mM Potassium ferricyanide, 50 mM sodium thiosuphate]

Potassium ferricyanide	0.05 g
Sodium thiosulphate	0.124 g

The solution was made up to 10 ml with MiliQ water and prepared prior to use.

100 mM ammonium bicarbonate

Ammonium bicarbonate	0.395 g

The solution was made up to 50 ml with MiliQ water.

Reducing solution [10 mM Dithiotreitol, 100 mM ammonium bicarbonate]

Dithiotreitol	0.015 g
Ammonium bicarbonate	10.00 µl

Dithiotreitol (DTT) was added to 100 mM ammonium bicarbonate prior to use.

Alkylation solution [55 mM Iodoacetamide, 100 mM ammonium bicarbonate]

Iodoacetamide	0.10 g
Ammonium bicarbonate	10.00 µl

Iodoacetamide was added to 100 mM ammonium bicarbonate prior to use.

Washing solution [50% (v/v) Acetonitrile, 100 mM ammonium bicarbonate]

Acetonitrile	25.00 ml
Ammonium bicarbonate	0.0395 g

The solution was made up to 50 ml with MiliQ water.

Digestion solution [7 ng/µl trypsin in 50 mM ammonium bicarbonate]

Trypsin	1.00 µl
Ammonium bicarbonate	749.00 µl

The solution was mixed together and made immediately prior to use.

2.6.2.2 In-Gel Trypsin digestion

Prior to in-gel digestion, the silver stained gel plug was destained by addition of 50 µl fresh destaining solution and shaked for 15 minutes. This step was repeated several times till yellowish colour of the gel plugs disappears. The gel piece was then reduced with reducing solution for 30 minutes at 60ºC. Then, it was alkylated with alkylation solution for 20 minutes at room temperature (RT) in dark. The gel plug was washed with 500 µl washing solution for 20 minutes. The washing step was repeated for three times.

The gel plug was then dehydrated by incubating in 50 µl of acetonitrile for 15 minutes. After removal of solution, the gel plug was left to dry using a speed vacuum Model-DNA 20 (Thermo Scientific, Rockford, USA). The dried gel plug was then incubated overnight at 37ºC in 25 µl of digestion solution. After spinning the sample down, 50 µl of 50% acetonitrile was added, vortexed and the sample was left at RT for 20 minutes. The supernatant was transferred into another eppendorf tube. A hundred percent of acetonitrile was then added to the gel plug in order to extract the remaining protein. The supernatant obtained was pooled with the previous supernatant. The supernatant was concentrated using the speed vacuum until the final volume become half from the initial volume.

The resulting peptide solution was desalted and concentrated using zip-tips (Perfect Pure C18, Eppendorf, Hamburg, Germany). The mass spectrometric analyses were performed by mixing 1 µl of extracted sample with equal volume of matrix solution

consisting of 10 mg/ml alpha-cyano-4-hydroxy cinnamic acid in 0.1% (v/v) trifluoroacetic acid and 70% (v/v) acetonitrile. The solution was finally spotted onto the slide loader and was allowed to air dry. The MALDI mass spectrometry was performed using the 4800 Plus MALDI TOF/TOF™ Analyser.

2.6.2.3 Database search

The Mascot programme (www.matrixscience.com) was used to search protein database. The programme uses peptide mass fingerprints (PMFs) to search the database for matching peptides from known proteins. The following parameters were used in the search: trypsin digests (one missed cleavage allowed), species: *Homo sapiens*, mass value: monoisotropic, peptide mass tolerance: $\pm$0.1 Da, peptide charge state: $\pm$ 1and SwissProt database. Identification was accepted when $\geq$5 peptide masses matched to a particular protein (mass error $\pm$50 ppm –1 missed cleavage) and the MOWSE score was over the threshold score at $p = 0.05$.

2.6.3 Gel-prep for mass spectrometry analysis by Australian Proteome Analysis Facility (APAF)

The replicate batch of gel plugs were also sent to APAF for protein identification using Coomassie Brilliant Blue R-250 staining as required by the facility. This was done to validate the protein identification done in the laboratory.

2.6.3.1 Stock solutions

Fixing solution [40% (v/v) methanol and 10% (v/v) acetic acid]

Methanol	100 ml
Acetic acid	25 ml

The solution was made up to 250 ml by addition of distilled water.

Staining solution [0.1% Coomassie Brilliant Blue R-250, 10% (v/v) acetic acid]

Coomassie Brilliant Blue R-250	0.25 g
Acetic acid	25 ml

The solution was made up to 250 ml by addition of distilled water.

Destaining solution [10% Acetic acid]

The solution was made up to 1 L by addition of distilled water.

2.6.3.2 Coomassie Brilliant Blue R-250 staining

All incubation steps of Coomassie staining were carried out with gentle agitation on a shaker. After completion of the electrophoresis run, the gel was immersed immediately in the fixing solution and left for at least 30 minutes. Fixing solution was then replaced with the staining solution and the gel was incubated overnight. Then, the gel was destained with a destaining solution. The destaining solution was changed several times until the proteins spots were stained with a clear background.

2.6.3.3 Preparation of gel plugs

Destaining solution [200 mM ammonium carbonate, 50% (v/v) acetonitrile]

Ammonium bicarbonate	0.79 g
Acetonitile	25 ml

The solution was made up to 50 ml by addition of MiliQ water.

Preserving solution [1% acetic acid]

Acetic acid	10 µl

The solution was made up to 1 ml by addition of MiliQ water.

The protein spots of interest were excised from the Coomassie stained gels and destained with 50 µl of destaining solution. The gel plugs were shaken for 15 minutes. This step was repeated several times until the bluewish colour of the gel plugs disappeared. The destaining solution was discarded and replace with 10 µl preserving solution. The gel plugs was kept in 1.5 ml tube (Eppendorf, Hamburg, Germany). The close cap of the tube was covered and tightens with parafilm. The gel plugs were then kept in -20˚C before being sent to APAF.

2.7 Western blotting

The Western blot was used as a validation purpose to further confirm the differentially excreted proteins found in patients with ovarian and cervical cancer. Detecting the spots with antibodies also validated the identity of the proteins determined using MALDI-TOF/MS. The samples were resolved using two-dimensional electrophoresis as described in Section 2.4 before it was being transferred onto a nitrocellulose membrane for Western Blotting analysis.

2.7.1 Stock solutions

Transfer buffer [48 mM Tris, 39 mM glycine, 20% (v/v) methanol]

Glycine	2.93 g
Tris	5.81 g
SDS	0.38 g
Methanol	200.00 ml

The mixture was dissolved and made up to 1 L with distilled water.

Tris-Buffered-Saline-Tween [100 mM Tris-HCl, pH 7.5, 0.9% Sodium chloride]

Tris	12.11 g
Sodium chloride	9.00 g

Tris was dissolved in 900 ml distilled water and the pH was adjusted to 7.5 with concentrated HCl. Sodium chloride and 0.1% (v/v) Tween-20 was then added before the solution was finally topped up to 1 L.

2.7.2 Protein transfer

NovaBlot Kit of Multiphor II Electrophoresis System (GE Healthcare, Uppsala, Sweeden) was used to transfer the proteins from 12.5% homogenous gel onto a nitrocellulose membrane (0.45 μm). Filter papers which were cut to the size smaller than the gel were soaked in the transfer buffer. Six layers of the wet filter paper were carefully layered one by one on a wet anode plate. The formation of bubbles between the layers was avoided. The nitrocellulose membrane was removed from its supporting film. The gel and the nitrocellulose membrane were soaked in the transfer buffer for a few minutes. The gel was then transferred onto the filter papers and the nitrocellulose membrane was placed on the top of the gel. Another six layers of filter paper were put on one by one on the gel. A

wet cathode plate was finally placed on the stack and the unit was run at 0.8 mA/cm², 600V for 1 hour except for CD59 protein which only required 30 minutes of transferring.

2.7.3 Development of protein spots

2.7.3.1 Stock solutions

Sodium phosphate buffer (0.01 M) pH 7.2

Sodium phosphate dibasic dihydrate	0.01 M
Sodium hydrogen phosphate	0.01 M
Sodium chloride	0.88% (w/v)

Sodium hydrogen phosphate and sodium chloride were dissolved in distilled water. The pH was adjusted to 7.4 using sodium phosphate dibasic dihydrate and the solution was made up to 1 L.

Sodium acetate buffer (1 mM) pH 4.4

Sodium acetate	8.02 g/L
Acetic acid	6.00 g/L

Three ml sodium acetate solution was topped up to 10 ml with acetic acid solution and the mixture was diluted 1:100 to give 1 mM buffer.

Sodium carbonate buffer

Sodium carbonate	21.2 g/L
Sodium hydrogen carbonate	16.8 g/L

Sodium carbonate solution was added to the sodium hydrogen carbonate to adjust the pH to 9.5.

Borate buffer

Disodium tetraborate	0.2 M
Boric acid	0.1 M

Approximately 115 ml borate solution was added to 4 L boric acid solution until the pH reached 7.4.

2.7.3.2 Enzyme conjugation of Champedak Galactose Binding (CGB) lectin

Futher confirmation of the altered level of fragment of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) was performed using HRP-conjugated CGB lectin. To prepare the conjugated CGB lectin, 4 mg of horseradish peroxidase (HRP) was dissolved in 1 ml distilled water. Two hundred µl of 0.1 M of freshly prepared sodium periodate was added to the solution. The solution was stirred gently for 20 minutes at room temperature before it was dialysed against 0.001 M acetate buffer pH 4.4 overnight at 4˚C. Twenty µl of 0.1 M sodium carbonate buffer pH 9.5 was added to the HRP solution to increase the pH to approximately 9-9.5. One ml of the lectin solution was immediately added and the solution was stirred for 2 hours at RT. One hundred µl of freshly prepared 0.1 M sodium borohydrate was added and the solution was left to stir at 4˚C for another 2 hours. The conjugated lectin was then dialysed against 0.1 M borate buffer pH 7.4. An equal volume of 60% (v/v) of glycerol in borate buffer was finally added to the solution. The solution was stored at 4˚C.

2.7.3.3 Detection of proteins of interest using specific antibodies and HRP-conjugated CGB lectin

After the transferring process, nitrocellulose membrane was left to dry for a while. The membranes were blocked with 3% w/v gelatine in Tris-buffered saline (TBS), pH 7.5,

for 1 hour at RT and washed three times with the same buffer. They were then incubated for another 1 hour in the following HRP-conjugate solutions: (1) anti-human CD59 (1:5 dilution), (2) anti-human kininogen-1 (1:500 dilution), (3) anti-albumin (1:40 dilution) and (4) champedak galactose binding (CGB) lectin (0.01 μg/ml) diluted/dissolved in TBST. The use of the CGB lectin to detect the C-terminal O-glycosylated ITIH4 fragment has been previously reported (Mohamed *et al*., 2008). Development of the Western blot was performed using 25 ng 3, 3'-diaminobenzidine and 5 μl of 30% v/v H_2O_2 in 50 ml TBS. The reaction was stopped by washing the membranes with distilled water.

2.8 SELDI-TOF-MS

In the last few years, several "gel-free" methods have been explored, and one of it is surface enhanced laser desorption/ionization-time of flight–mass spectrometry (SELDI-TOF-MS), ProteinChip Biomarker System. This is a unique approach in which peptides are separated on the target itself by virtue of their chromatographic properties. The sample drawn from the patient is applied to a protein chip, which is made up of a specific chromatographic surface. After several washing steps and the application of an energy-absorbing molecule, the species that are retained on the surface of the chip are analysed via mass spectrometry. SELDI-TOF-MS is particularly appropriate for the investigation of low molecular weight proteins (<20 kDa) with femtomole sensitivity and the ability to examine native proteins as a complementary methods to better elucidate the protein composition of biological samples (Baggerman *et al*., 2005). The same individual samples of control (n=11), OCa (n=8) and CCa (n=8) which was previously analyzed with 2-DE were subjected to the SELDI-TOF-MS analysis.

2.8.1 Stock solutions

Binding buffer for protein chip CM 10 array [100 mM NH_4Ac, pH 4.4, 0.05% triton]

Ammonium acetate	0.3854 g
Triton	25 µl

The ammonium acetate solution was made up to 50 ml with MiliQ water and the pH was adjusted to 4.4 before triton was added.

Binding buffer for protein chip Q 10 array [100 mM Tris-HCl, pH 9, 0.05% Triton]

Tris	0.6057 g
Triton	25 µl

The Tris solution was made up to 50 ml with MiliQ water and the pH was adjusted to 9 before Triton was added.

Alpha-cyano-4hydroxycinnamic acid (CHCA matrix solution) [CHCA, 50% Acetonitrile, 0.25% Trifluoroacetic acid]

CHCA	5.00 mg
Acetonitrile (ACN)	100.00 µl
Trifluoroacetic acid (TFA)	0.50 µl

The ACN and TFA were made up to 200 µl with MiliQ water before it was added to a vial containing manufacturer's CHCA. The mixture was centrifuged and the draw off supernatant was ready to be used after dilution with equal part of the similar solution.

Sinapinic acid (SPA matrix solution) [SPA, 50% ACN, 0.25% TFA]

SPA	5.00 mg
Acetonitrile	200.00 µl
Trifluoroacetic acid	2.00 µl

The ACN and TFA were made up to 400 µl with MiliQ water and were added to the manufactured SPA vial. The solution was then centrifuged. The solution was prepared prior to use. All the matrix types were prepared according to the manufacturer's protocol.

2.8.2 Chip preparation to be used in SELDI-TOF-MS

The type of chips used in this analysis were the ProteinChip® CM10 arrays and ProteinChip® Q10 arrays (Biorad Laboratories, Hercules, USA). The ProteinChip® CM10 Arrays incorporates a carboxylate chemistry (negatively charged) and thus acts as a weak cationic exchanger. Therefore, ProteinChip ® CM10 array surface binds protein that are positively charged at a given pH, whilst for the ProteinChip® Q10 array incorporates quarternized ammonium groups (positively charged) and thus acts as strong anion exchanger. The surface of Q10 chip binds peptides and proteins that are negatively charged at a given pH. These two types of protein chip were chosen to get an optimum binding of the whole protein in the urine sample since previous researcher had demonstrated high yield of protein recovery using these chips compared to the others protein chip (Papale *et al.,* 2007 and Roelofsen *et al.,* 2007)

The protein chip array cassette (CM10 or Q10) was first placed in the bioprocessor. Two hundred µl binding buffer was added to the bioprocessor. The binding buffer was incubated for 5 minutes with agitation at 250 rpm. The buffer was then removed from the wells and replaced by a mixture of 160 µl binding buffer and 40 µl urine samples (equivalent to 80 µg proteins dissolved in MiliQ water). The solution was incubated for 30

minutes and the bioprocessor was covered with parafilm to avoid any contamination. After the incubation, the sample was removed and washed three times for 5 minutes with 200 µl binding buffer. Then, 200 µl of Mili Q water was added to the bioprocessor. The water was removed and this step was repeated once. Then, the array was left to dry and 1 µl of matrix solution was applied onto each spot on the chip. The spots were left to dry and the matrix solution was reapplied once more. The CHCA matrix solution was used for ProteinChip® CM10 whereas the SPA matrix solution was used with ProteinChip® Q10. Mass accuracy was calibrated on the day of analysis using the All-in-one peptide molecular mass standard (Ciphergen Biosystems, Inc., Fremont, CA, USA).

2.8.3 Reproducibility of the spectra

The reproducibility of protein spectra such as mass location and intensity between chips was determined from the control urinary protein samples analysed in the study. The protein peaks were randomly selected from five control urine samples. The selected peaks with *m/z* range of 16.742 – 16.758 and 9906.07 – 9913.98 were used as a quality control (QC) to calculate the coefficient of the variance (CV). The CV of peak location and normalised intensity were determined before the urinary protein of the control and both cancer patients were analysed.

2.8.4 SELDI-TOF-MS Data Analysis

2.8.4.1 Chipergen Express Data Manager

All the chips were read by adopting the same protocol (acquisition mode: source at 25 kV-Positive Ions, laser energy 1500 nJ, matrix attenuation 500, focus mass 20,000 Da, sample rate 800 MHz, partition 1 of 4, acquired mass range from 5000 to 30,000) in the ProteinChip Biomarker System. ChiphergenExpress Data Manager is an application within

ProteinChip Biomarker System used to generate peak cluster and export these clusters into a file for analysis in Biomarker Patterns^{TM} Software (BPS). The software consists of Cluster Wizard and P-Value Wizard. In order to export a file to BPS from CiphergenExpress Data Manager, a new cluster was created. Since the present study used two different ProteinChip® array (CM10 and Q10), the set of spectra was first specified base on the array type condition by selecting the 'array type' fields in the Cluster Wizard.

CiphergenExpress Data Manager operates in two passes. The first pass uses low sensitivity to detect obvious and well-defined peaks, and the second pass uses low sensitivity settings to search for smaller peaks, with the mass values found in the first pass. The following parameters were used in specifying the clustering parameter for the peak detected (First pass: 5 S/N, Minimum peak threshold: 20% of all spectra) and cluster completion (Cluster Mass Window: 0.3% of Mass, Second Pass: 2 S/N). In order to compare control, ovarian and cervical cancer patients; the sample group was chosen as a field for groupings to be used in the *p*-value calculation under the P-Value Wizard. The system then prompts for a file name and location for saving the file in the .csv format. The variable heading for the .csv file generated by the ChiphergenExpress Software was labelled automatically to indicate the conditions by names starting with C0. The data in .csv format was then ready to be run with BPS.

2.8.4.2 Biomarker Pattern Software 5.0

The data obtained from CiphergenExpress Data Manager was finally analysed using Biomarker Pattern Software 5.0 (BPS). Implementation of CART (**C**lassification **a**nd **R**egression **T**rees), a statistical procedure introduced by Leo Breiman, Jerome Friedman, Richard Olshen, and Charles Stone in 1984 eliminates the potential problem of 'overfitted data', a condition that occurs when many data points are analyzed against a relatively small

sample set, a situation frequently encountered in the early phases of a discovery project, when a relatively small number of samples may be available.

As the name suggests, BPS is a single procedure that can be used to analyze either categorical (classification) or continuous (regression) data using the same technology. However, for SELDI-TOF-MS in this study, only classification data was used. A defining feature of CART is that it presents its results in the form of decision trees. The tree structure allows BPS handles massively complex data while producing diagrams that are easy to understand.

BPS begins with a **root node** (Node 1) and, through a process of yes / no questions, it generates descendant nodes. Some nodes are **terminal**; meaning that a final classification is reached and further splitting has no gain in data classification. The splitting decisions in this case were based on the normalized intensity levels of peaks from the SELDI protein expression profile. Each peak or cluster identified from the SELDI profile is therefore a variable in the classification process. Multiple classification trees were generated using this process, and the best-performing tree was chosen for testing. During the analysis, a pruning step occurs in which branches are removed and the cost of the removal is determined to establish a minimal tree size. This is referred to as a "learning set".

The decision tree was then subjected to cross validation. In this step, the data are partitioned such that randomly selected samples are categorized to ensure that the decision tree is valid. Cross validation is used if data are insufficient for a separate test sample. In such cases, BPS grows a maximal tree on the entire learning sample. This is the tree that will be pruned. BPS then proceeds by dividing the learning sample into 10 roughly equal parts, each containing a similar distribution for the dependent variable. It takes the first nine parts of the data, constructs the largest possible tree, and uses the remaining tenth of the

data to obtain initial estimates of the error rate of selected sub-trees. The same process is then repeated (growing the largest possible tree) on another 9/10 of the data while using a different tenth part as the test sample. The process continues until each part of the data has been held in reserve one time as a test sample. The results of the 10 mini-test samples are then combined to form error rates for trees of each possible size; these error rates are applied to the tree based on the entire learning sample. The upshot of this complex process is a set of fairly reliable estimates of the independent predictive accuracy of the tree. This allows determination of how well any tree will perform on completely fresh data, even if there is no independent test sample. Because the conventional methods of assessing tree accuracy can be wildly optimistic, cross validation is the method BPS normally uses to obtain objective measures for smaller data sets.

In the present study, each split separated a parent node into exactly two child nodes.The terminal nodes of the peak profile were validated against the *p*-value generated from the ChiphergenExpress Data Manager. A *p*-value of less than 0.05 was considered to be significantly difference.

2.8.4.3 Sensitivity and specificity of the protein of interest

In medical diagnostics, test sensitivity is the ability of a test to correctly identify those with the disease (true positive rate), whereas test specificity is the ability of the test to correctly identify those without the disease (true negative rate). In this study, the sensitivity was obtained directly from the BPS software. However, the specificity was calculated based on the standard formula as shown as follow (Altman *et al.*, 1994):

		Actual Condition	
		Positive	***Negative***
Test outcome	***Positive***	**True Positive**	**False Positive** (Type I error)
	Negative	**False Negative** (Type II error)	**True Negative**
		↓ **Sensitivity** $= \left(\frac{\Sigma \text{ True Positive}}{\Sigma \text{ Condition Positive}} \right) \text{x}100$	↓ **Specificity** $= \left(\frac{\Sigma \text{ True Negative}}{\Sigma \text{ Condition Negative}} \right) \text{x}100$

"Σ Condition positive" is the sum of true positive and false negative whereas "Σ Condition negative" is the sum of false positive and true negative.

2.9 Statistical analysis

All values are presented as mean ± SD (standard deviation). However, the reproducibility assessment of the 2DE gels and blots were presented as RSD (relative standard deviation). In Image Master 2D Platinum analysis, the percentage of volume contribution between control subjects and patients were determined by the software. The Student t-test was then used to analyse the significance of differences between control subjects and patients base on the percentage of volume contribution. A *p*-value of less than 0.05 was considered significant. Implementation of CART in BPS, a classification statistical procedure was specifically used for the SELDI-TOF-MS analysis.

Chapter 3
RESULTS

3.1 Two-dimensional electrophoresis urinary protein profiles

Typical unidimensional SDS-PAGE separates proteins by their size. The drawback of this method is if the protein with the similar molecular weight were subjected for separation under the same electrophoresis, they would overlap and appear as a single band on the gel. 2-DE offers a better resolution and separation of the protein where they are separated according to their pI for the first dimension, and their molecular weights for the second dimension. By introducing several manipulations to the general protocol of 2-DE separation, the optimum condition for the separation and the reproducibility of the separation technique of urinary proteins were achieved.

The 2-DE technique performed on ovarian cancer patients (OCa), cervical cancer patients (CCa), and cancer negative women (control) were separated and compared. Reproducibility of the 2-DE separation technique was assessed by carrying out the analysis of each sample in duplicate. Separation of urine protein samples by 2-DE resulted in highly resolved profiles comprising more than 10 clusters of protein spots. Figure 4 demonstrates a representative urinary proteome profile obtained from a control subject. Seven protein spot clusters consistently appeared in all the 15 control samples analyzed and there was no apparent difference in the intensity of the spots between the individual urine samples studied.

3.2 Typical urinary protein profiles of healthy control women and patients with OCa and CCa

Urine samples of control healthy female controls, OCa patients and CCa patients were subjected to 2DE and silver staining. When the gel-based proteomic analysis was performed on urinary protein samples from OCa patients (n = 11), different 2-DE profiles were obtained (Figure 5, panel A).

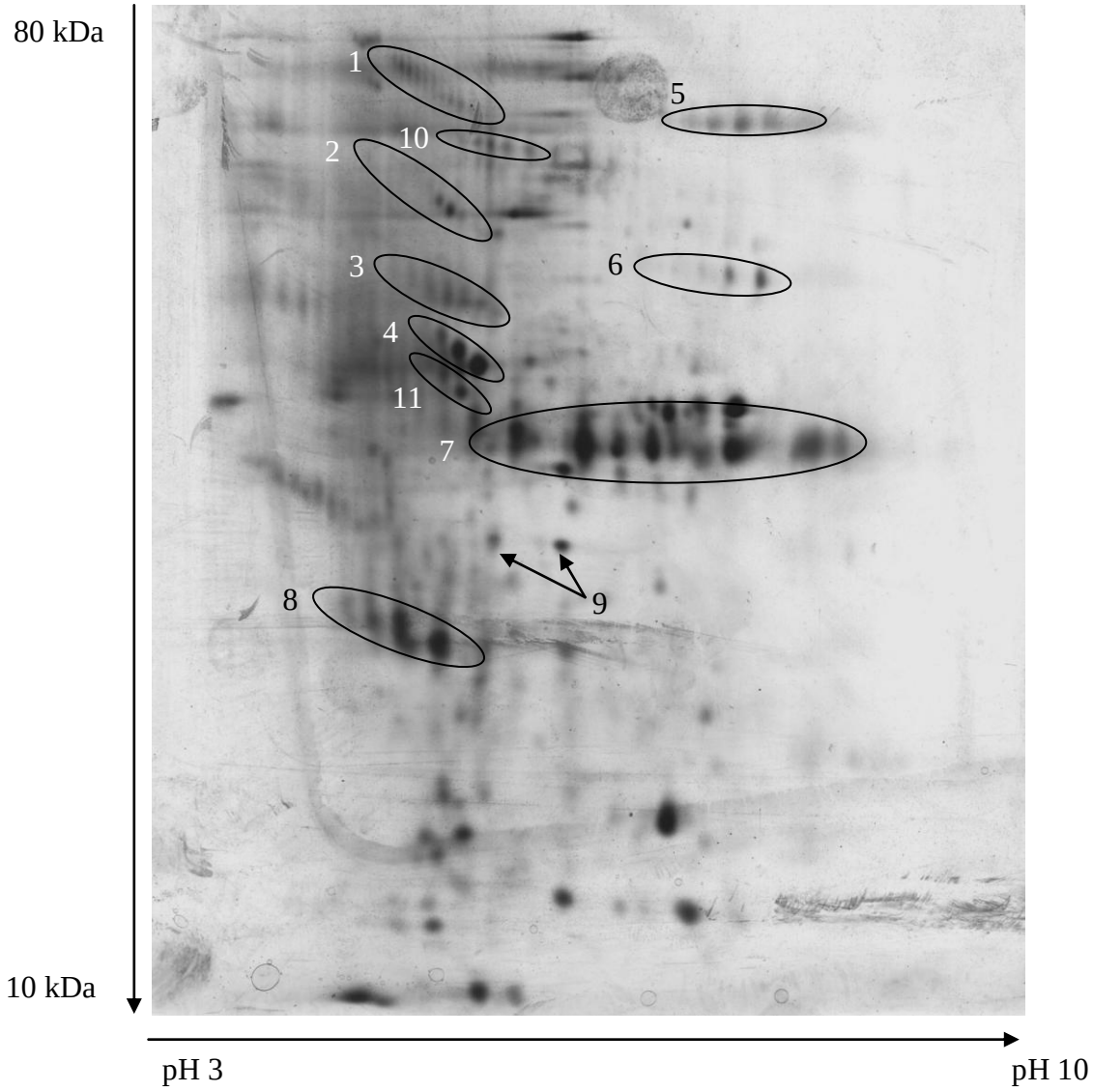


Figure 4: Typical 2-DE map of urinary proteome from control subject generated by Silver Staining.

Three hundred µg of purified urinary protein was dissolved with 200 µl rehydration solution before subjected to 2-DE using immobiline drystrip pH 3-10 followed by silver staining. Seven protein spot clusters consistently appeared in all the 15 control samples analyzed which had no apparent difference in the intensity of the spots between the individual urine samples studied were marked as number 2, 4, 5, 6, 7, 10 and 11. Cluster 1, 3 and 8 are KNG1, ITIH4f and CD59, respectively.

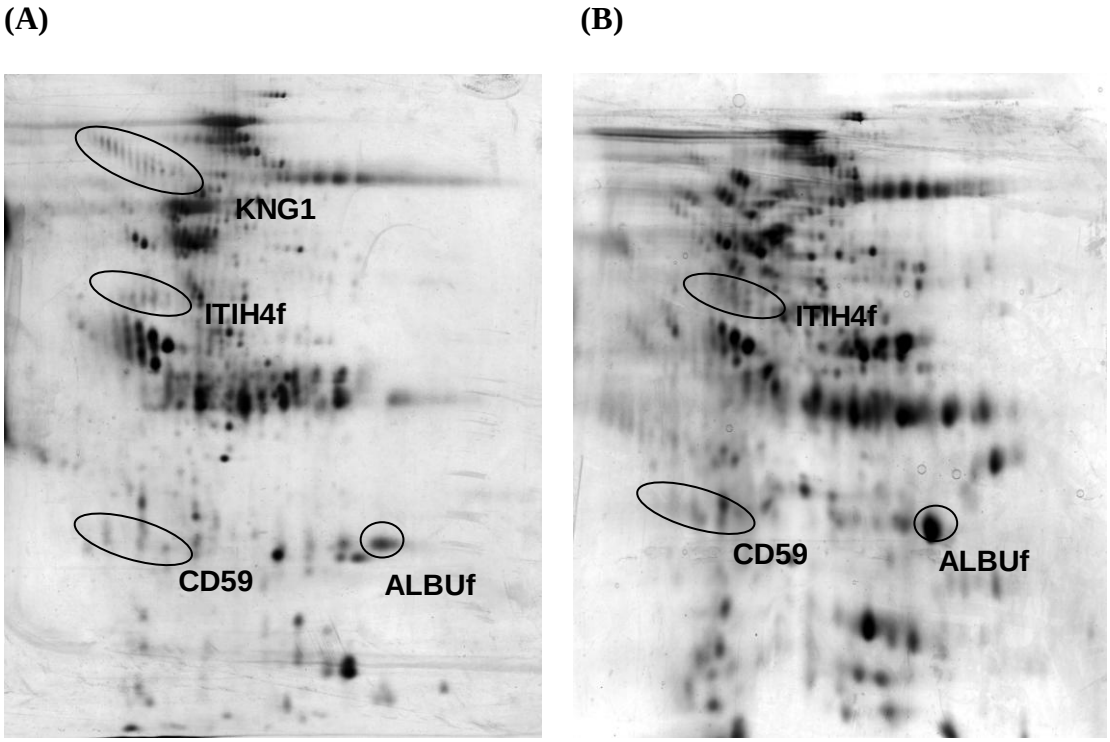


Figure 5: Typical 2-DE urinary protein profiles of patients with OCa and CCa.

Panels A and B demonstrate the representative 2-DE urinary protein profiles of ovarian cancer patients (n=11) and cervical cancer patients (n=11), respectively. The aberrantly excreted urinary protein spot clusters were marked in circles. ITIH4f and ALBUf refer to fragments of inter-alpha-trypsin inhibitor heavy chain H4 and albumin, respectively. In contrast to the ovarian cancer patients, KNG1 was found to be insignificantly different when compared to the control subject although the KNG1 protein cluster pattern was much alike to that of ovarian cancer patients.

Three protein spot clusters which consistently appeared in the control profile were either not detected or were reduced in intensity in the cancer patients in a considerable number of the patients' 2-DE gels. The levels of the other protein spot clusters were comparable to those detected in the urinary proteome profiles of the control subjects.

Similar to the ovarian cancer, two out of three protein spots were also found to be reduced or not detected in the cervical cancer patients (Figure 5, panel B). One protein spot appeared to be enhanced in both ovarian and cervical cancer patients. However, there were no significant abnormal expressed proteins found on the gels when comparing the ovarian cancer patients to cervical cancer patients.

3.3 Image analysis of 2-DE gels

The different altered levels of CD59, kininogen-1 and fragments of ITIH4 and albumin in the urine of patients with ovarian carcinoma and cervical cancer, relative to the controls, was confirmed when their 2-DE urine protein profiles were subjected to image analysis using the Image Master 2D Platinum Software 7.0. Image analysis also confirmed that the levels of the other highly resolved urine protein spot clusters were comparable between cancer patients and controls. Figure 6 demonstrates the mean percentage of volume contribution of the four urine proteins of interest in control subjects and patients with ovarian carcinoma. When taken as overall, the levels of CD59, KNG-1 and ITIH4 fragment were significantly lower in ovarian carcinoma patients by 4-, 2- and 2-folds, respectively, compared to those excreted by the control subjects. In contrast, the 19 kDa fragment of albumin appeared 10-fold higher in the patients' urine.

Meanwhile, the mean percentage of volume contribution of the three urine proteins of interest in control subjects and patients with cervical cancer was demonstrated in Figure 7.

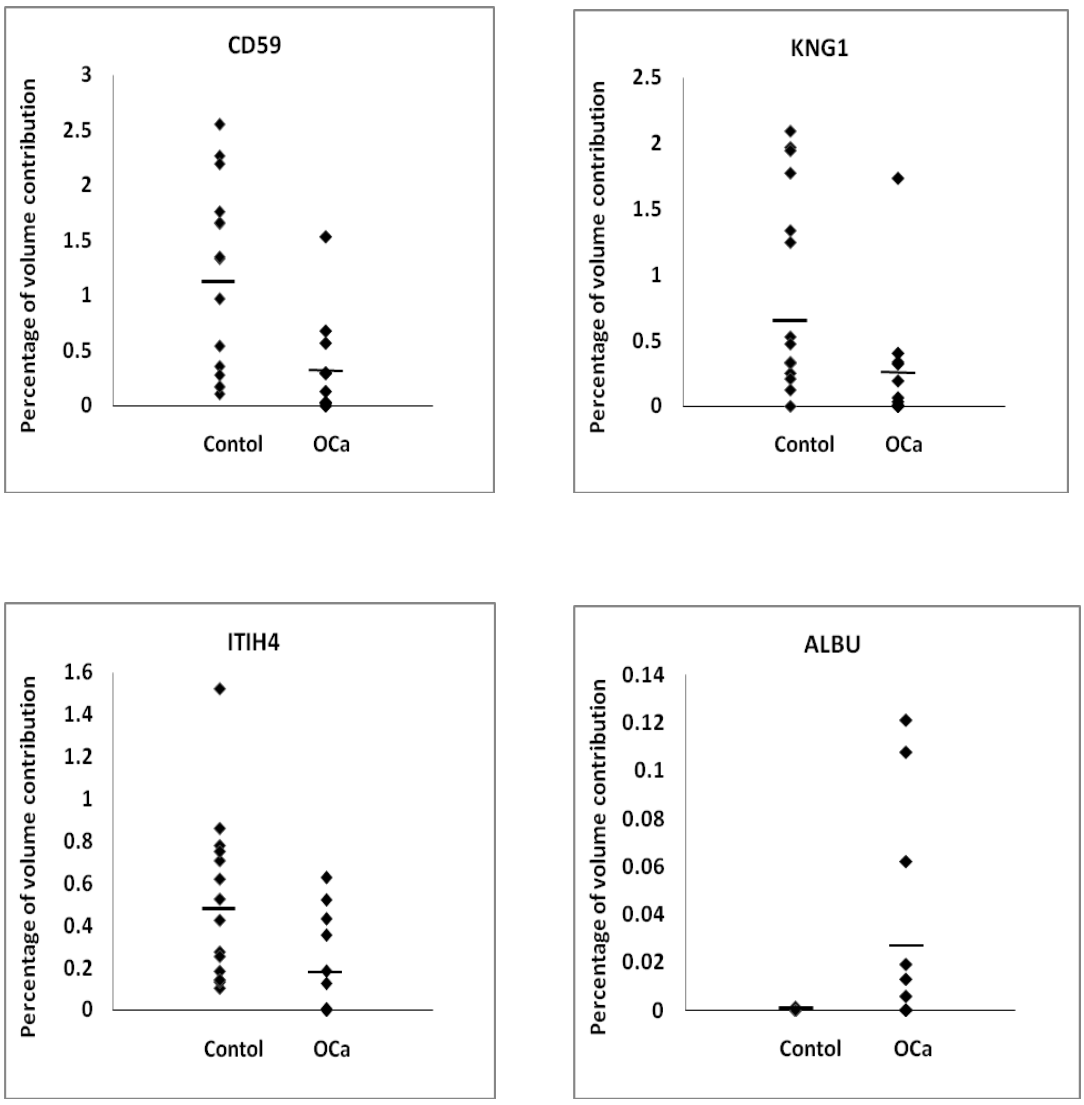


Figure 6: Relative excretion of urinary proteins obtained from control subjects and patients with OCa.

The percentage of volume contribution was determined using the Image Master 2D Platinum Software 7.0. Image analysis performed on protein spot clusters that appeared consistently within each cohort of urine samples demonstrated the aberrant excretion of CD59, kininogen-1 (KNG1), ITIH4 (39 kDa fragment) and 19 kDa fragment of albumin in patients with ovarian carcinoma.

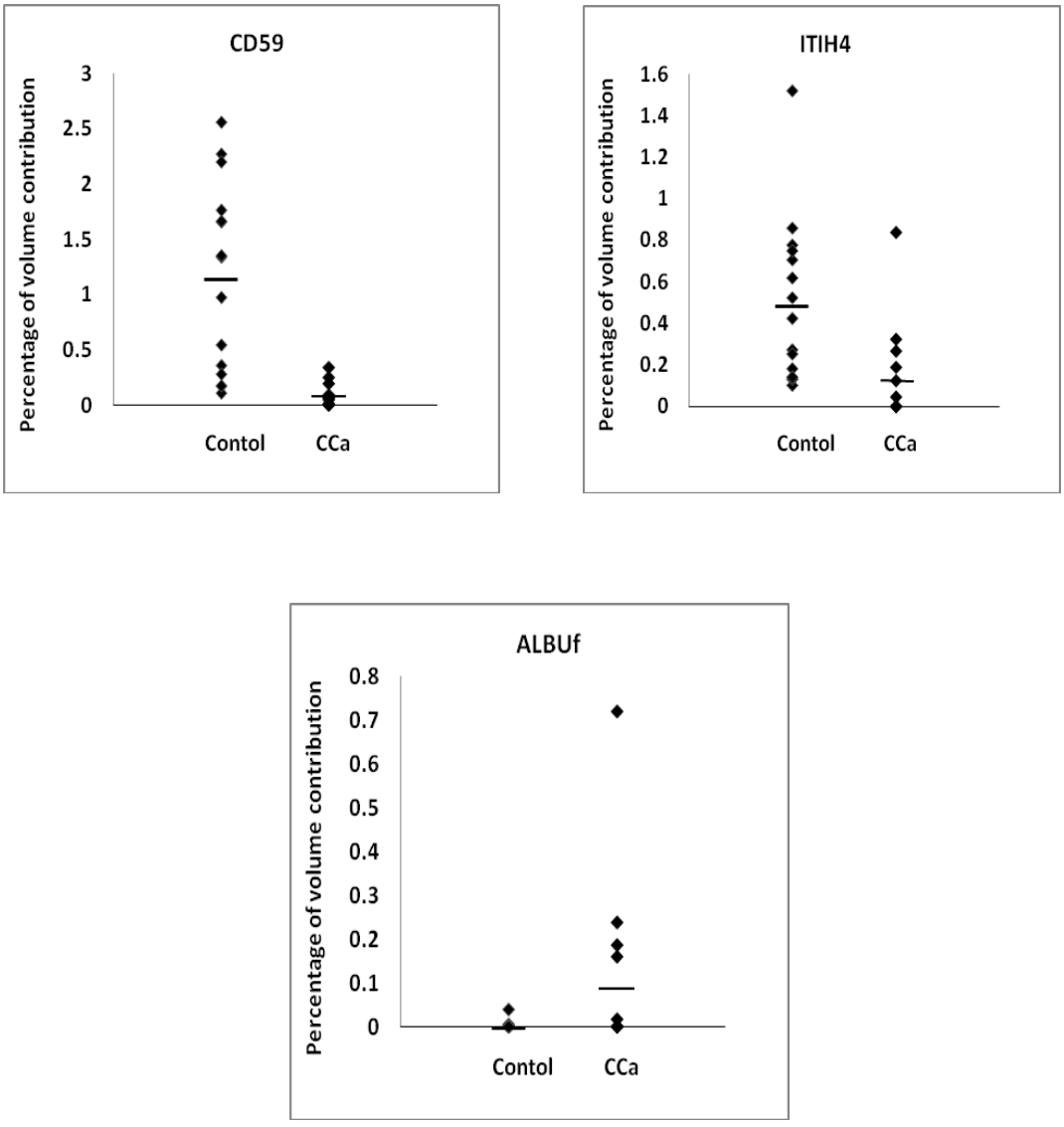


Figure 7: Relative excretion of urinary proteins obtained from control subjects and patients with CCa.

The percentage of volume contribution was determined using the Image Master 2D Platinum Software 7.0. Image analysis performed on protein spot clusters that appeared consistently within each cohort of urine samples demonstrated the aberrant excretion of CD59, ITIH4 (39 kDa fragment) and 19 kDa fragment of albumin in patients with cervical cancer.

When taken as overall, the levels of CD59 and ITIH4 fragment were significantly lower in cervical cancer patients by 11- and 3-folds, respectively, compared to those excreted by the control subjects. In contrast, the 19 kDa fragment of albumin appeared 60-fold higher in the patients' urine (Table 3). The fragment of albumin was expressed in bulk in certain patients of cervical cancer.

3.4 Identification of Aberrantly Excreted Urinary Proteins

Subjecting the spot clusters of urine proteins that were aberrantly excreted to mass spectrometry and database search identified them as CD59, kininogen-1, inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and albumin. Table 4 shows a summary of the data acquired. High probability-based MOWSE scores were obtained for all the urinary proteins. Results sent to APAF for comparison purpose of proteins identification were tallied with in-house experiment. Among the four urinary proteins of interest, ITIH4 and albumin demonstrated large discrepancies between the experimental masses that were estimated based on their mobilities in the 2-DE gels and their theoretically calculated mass. This suggests that the ITIH4 and albumin spots detected in the 2-DE urinary profiles were truncated fragments of their native molecules.

Table 5 demonstrated the list of matched peptide sequences with high confidence, by MS/MS analysis. Sequences of peptide obtained from the MALDI-TOF-MS were then compared against CD59 (P13987; 128 amino acid), KNG1 (P01042; 644 amino acid), ITIH4 (Q14624; 930 amino acids) and albumin (P02768; 585 amino acids) in the Swiss-Prot database. Based on the sequence and the experimental molecular weight, it is believed that ITIH4 and albumin are fragments of their respective proteins.

Table 3: Relative expression of urinary proteins analyzed by 2-DE.

Urinary proteins	Analysis by 2DE			
	OCa patients		CCa patients	
	Fold changes*	*p*	Fold changes*	*p*
CD59	- 3.60	6.3×10^{-3}	- 10.95	3.95×10^{-4}
Kininogen-1	- 2.13	3.95×10^{-2}	n.s.	n.s.
ITIH[tf]	- 2.16	4.11×10^{-2}	- 2.79	2.21×10^{-2}
Albumin[tf]	+ 10	3.58×10^{-2}	+ 60	4.7×10^{-2}

*Fold expression changes were relative to that of control values

(–) decrease in expression; (+) increase in expression

[tf] Truncated fragment of protein

"n.s." not significant

A *p* value of less than 0.05 was considered significant.

Table 4: Mass spectrometric identification of spot clusters from urinary protein profiles.

Protein	Accession number[#]	Nominal mass (kDa) / p*I*	Mean Experimental mass* (kDa)	In house MS analysis		APAF MS analysis	
				MOWSE protein score	Sequence coverage (%)	MOWSE protein score	Sequence coverage (%)
CD59	P13987	14.168 / 6.02	19.43	160	18	108	28
KNG1	P01042	71.912 / 6.34	64.34	193	15	97	39
ITIH4	Q14624	103.262 / 6.51	38.85	120	7	59	2
ALBU	P02768	69.321 / 5.92	19.45	161	8	107	28

Spot ID are as in Figure 5. KNG1, ITIH4 and ALBU refer to kininogen-1, inter-alpha-trypsin inhibitor heavy chain H4 and albumin, respectively.

[#] Accession numbers are from the Mascot database (www.matrixscience.com).

* Estimation based on migration in the 2-DE gels relative to protein markers.

Table 5: In house list of matched peptide sequences of high confidence identified from MS/MS analysis.

Peptide sequence	Ion score	Amino acid
39 kDa ITIH4 spots		
R.QGPVNLLSDPEQGVEVTGQYER.E	19	754 - 775
K.WKETLFSVMPGLK.M	31	814 - 826
R.RLDYQEGPPGVEISCWSVEL.-	24	911 - 930
19 kDa albumin spots		
K.QEPERNECFLQHK.D	30	118 - 130
K.DDNPNLPR.L	37	131 - 138
K.YLYEIAR.R	43	162 - 168
K.LDELRDEGK.A	10	206 - 214
K.VHTECCHGDLLECADDR.A	41	265 - 281
KNG1 spots		
K.TWQDCEYKDAAK.A	46	90 - 101
R.QVVAGLNFR.I	54	188 - 196
K.KYFIDFVAR.E	69	316 - 324
K.YFIDFVAR.E	45	317 - 324
CD59 spots		
K.FEHCNFNDVTTR.L	85	67 – 78
R.LRENELTYYCCK.K	61	79 – 90

Sequences of peptide obtained from the MALDI-TOF-MS were checked against CD59 (P13987; 128 amino acid), KNG1 (P01042; 644 amino acid), ITIH4 (Q14624; 930 amino acids) and albumin (P02768; 585 amino acids) in the Swiss-Prot database.

3.5 Western blotting

Further confirmation of the altered levels of CD59, kininogen-1 and fragments of ITIH4 and albumin in the urine of patients with ovarian carcinoma relative to those of the control subjects was performed using antisera and a lectin that bind to the respective proteins that were blotted onto membranes. Figure 8 demonstrates the respective interactions of specific antisera and the CGB lectin with the four proteins of interest in pooled urine samples of patients and control subjects. In case of the 19 kDa albumin fragment, interaction appeared to be detected only in the pooled urine of patients with ovarian carcinoma compared to that of the controls, while the inverse was observed for CD59, kininogen-1 and the 39 kDa fragment of ITIH4.

3.6 SELDI-TOF-MS analysis

Similar to the previously reported SELDI-TOF-MS analysis (Petricoin *et al.* 2002; Adam *et al*. 2002), the present analysis of urinary proteins from control, OCa patients and CCa patients could be divided into three steps: peak detection and alignment, selection of peaks with the highest discriminatory power and lastly data analysis using a decision tree algorithm. OCa (n=8) and CCa (n=8) urine samples, that had been previously analyzed using two-dimensional gel electrophoresis, were used in this SELDI-TOF-MS analysis. Samples from controls were used as a comparison in identifying peaks corresponding to aberrantly expressed proteins in both cancers.

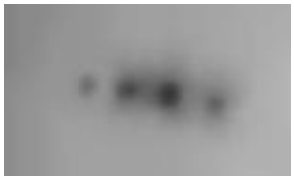
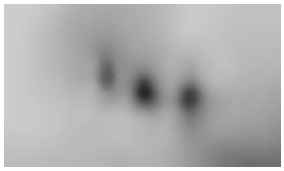
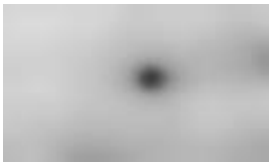
Antiserum/lectin versus	Control	OCa	CCa
kininogen-1 (~64 kDa)			Not significant
ITIH4 (~39 kDa)			
CD59 (~19 kDa)			
albumin (~19 kDa)			

Figure 8: Interaction of antisera and CGB lectin with aberrantly excreted urinary proteins of OCa and CCa patients.

Pooled urine samples of OCa patients, CCa patients and those of control subjects were subjected to 2-DE and Western blotting before being independently exposed to antisera that bind to CD59, kininogen-1 and albumin as well as the ITIH4-binding-CGB lectin. As for the CCa, kininogen-1 was not further tested since the Image Master has indicated that the result was not significant.

3.6.1 Determination of the reproducibility of the spectra

The reproducibility of protein spectra such as mass location and intensity between chips was determined using the control samples. Before the analysis is done, it is important to verify the reproducibility of the peaks since further investigation would depend on the location and intensity of peak spectrum. Spectral reproducibility was evaluated according to relative intensity *m/z*. The selected peaks with *m/z* range of 16.742 – 16.758 and 9906.07 – 9913.98 were used to evaluate the reproducibility of the spectra.

The protein peaks were randomly selected from five control samples that were analysed in a same day using Protein Chip® CM10 array and Protein Chip® Q10 array. The coefficient of variance (CV) for peak location (*m/z*) and normalised intensity were 0.04% and 17%, respectively for Protein Chip® CM10 whereas the CV for Protein Chip® Q10 were 0.04% and 19%, respectively. Generally CV less than 20% was observed in peak intensity from human data analysed using SELDI-TOF-MS since peak intensity was typically more variable in many biological assays (Poon *et al.*, 2007). These low percentages of CVs obtained from peak location and peak intensity in this study were within a good range and indicating high repeatability and thus validating the reproducibility of the peak spectra.

3.6.2 Establishment of a diagnostic decision tree

3.6.2.1 Evaluation of the classification tree quality

Figure 9 is the representative of the control group, where the protein mixture was separated using ProteinChip® CM10. As soon as BPS has completed its calculation, it opens a window containing the resulting calculation tree topology with a visual indication of the tree quality (Figure 9). The tree was made of nodes with different colour ranging from red to blue where red is defined as better quality of classification.

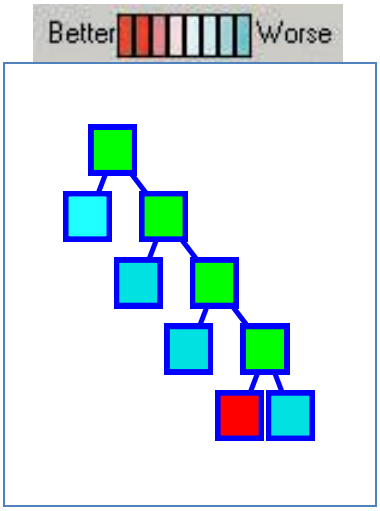


Figure 9: A Representative of a classification tree topology for the control group.

Control group was selected at the software navigator in order to view the classification quality. While the BPS was commanded to grow or prune the tree, the colour of the terminal node will change and indicate the quality of classification. The red is defined as better quality of classification and the blue as worse. The decision node is where the splitting occurred and designated as green squares. The nodes where the splitting ends at the end of each branch are known as terminal node.

As an example in Figure 9, by selecting the control group at the software's navigator command, the software will present a red terminal node in which the majority of cases selected in the nodes were the control samples. While the BPS was commanded to grow or prune the tree, the color of the terminal node will change and indicate the quality of classification. Thus, the tree quality which is its ability to differentiate between the three cohorts, could be evaluated by using the software's navigator command.

3.6.2.2 Cross validation

Cross validation is a statistical method of evaluating and comparing learning algorithms by dividing data into two segments: one used to learn and train a model and the other used to validate the model (Payam *et al.*, 2009). As the classification tree built in this study, may demonstrate adequate prediction capability on the learning data, it might fail to predict future unseen data (Larson, 1931). Although the number of sample in this study was low, it was enough to construct the classification tree. However, a blind test could not be performed to validate the constructed tree, but the performance of the tree could still be estimated by having cross validation procedure calculated automatically by the BPS.

The BPS started with growing the tree to the largest maximal tree possible and prunes away until it finds the best tree. The resulting decision tree which referred as 'learning' set was then subjected to cross validation. In this step, the data are partitioned such that randomly selected samples are categorized to ensure that the decision tree is valid. The error curve graph shown in Figure 10 demonstrates the cross-validated relative cost or misclassification cost generated by the application while pruning the tree. It indicated a good classification was obtained where the graph demonstrated the rate of misclassification to go down as the tree gets progressively larger.

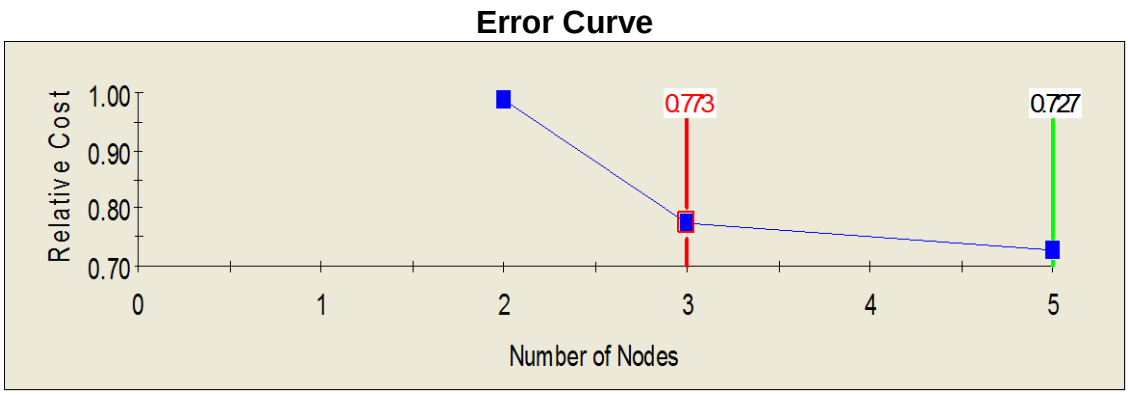


Figure 10: A representative of error curve from control group

An error curve graph of the control group displaying the cross validated relative cost versus the number of terminal nodes for each tree size in the nested sequence. The cost validated relative cost is known as the misclassification cost generated while pruning the tree. The misclassification rate went down as the tree gets progressively larger.

3.6.3 Identification of the biomarker pattern and the construction of diagnostic model separate by ProteinChip® CM10

Four peaks, with *m/z* of 8828.8, 7528.7, 5393.9 and 6176.7 in Figure 11 were the best determinant of the decision tree. At Node 1 (defined by peak 8828.8), one control, one ovarian cancer patient and seven cervical cancer patients with peak intensities lower than 26.199 μA were classified into Terminal Node 1. The other 18 samples were further classified at Node 2 (defined by peak 7528.7), where six ovarian cancer patients with peak intensities of 34.199 μA or less were classified to Terminal Node 2. The other 12 samples entered Node 3 (defined by peak 5393.8), where one cervical cancer patient with peak intensities of 3.644 μA or less went to Terminal Node 3. The other 11 samples were further classified at Node 4 (defined by peak 6176.7), where ten controls with peak intensities of 75.928 μA or less went to Terminal Node 4. The remaining one sample, which was from ovarian cancer patient, was classified to Terminal Node 5. Overall, the model correctly classified 10 out of 11 control and seven out of eight ovarian cancer samples. Interestingly, it was abled to classify all eight cervical cancers samples correctly.

3.6.4 Identification of the biomarker pattern and the construction of diagnostic model separate by ProteinChip® Q10

Figure 12 showed four peaks with *m/z* of 5937.1, 6186.2, 6349.5 and 15802 which were the best determinant of the decision tree. At Node 1 (defined by peak 15802), five ovarian cancer patients with peak intensities higher than 14.468 μA were classified into Terminal Node 6. The other 22 samples with peak intensities lower than 14.468 μA were further classified at Node 2. The population was further divided based on the peak intensity of 4.482 μA. Interestingly, all five samples which fall under Terminal Node 5 were from cervical cancer cohort. The remaining 17 samples were further classified at Node 3

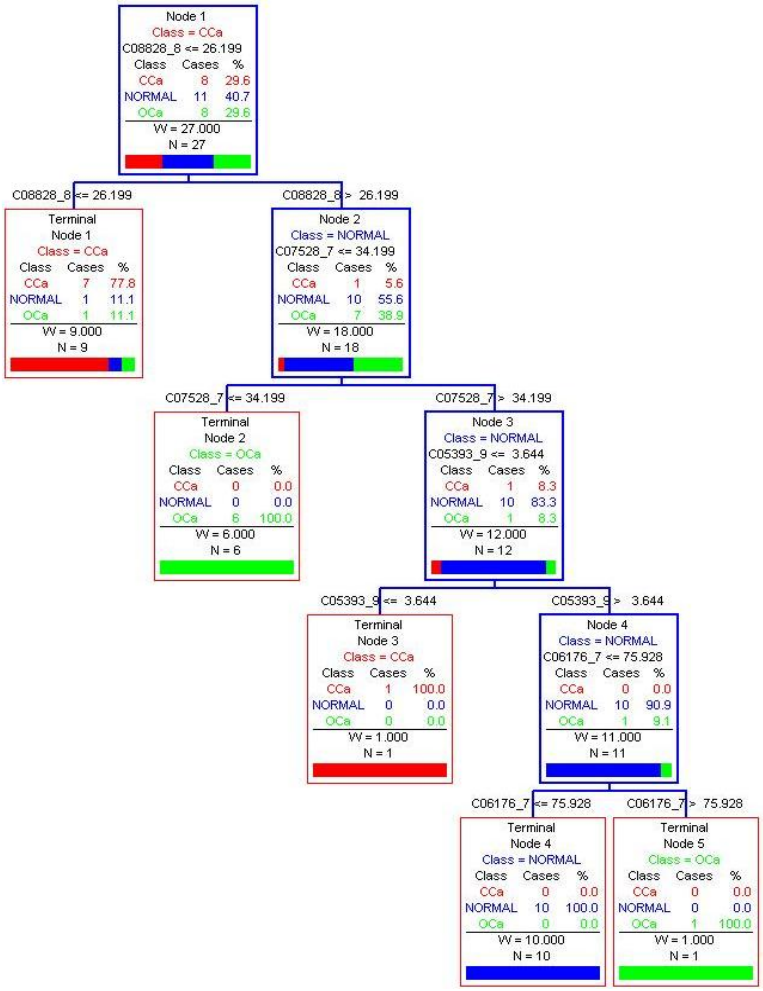


Figure 11: The decision tree of samples upon separation of protein by ProteinChip® CM10 arrays.

A tree structure of the classification model was generated to classify the OCa, CCa and control samples based on the peaks separated by the ProteinChip® CM10 Arrays. In the hierarchy rectangles, the presence or absence of three SELDI-TOF-MS peaks, with *m/z* value of 8828.8, 7528.7, 5393.9 and 6176.7 serves as parent nodes to classify samples in a hierarchical way. The threshold value which was shown by the *m/z* value following the peak intensity was where the splitting occurred. Samples were classified into a lower level node until the final classification was reached. For example, C08828_8, which is the intensity of the peak, observed in the data indicated the conditions of variable for *m/z* 8828.8.

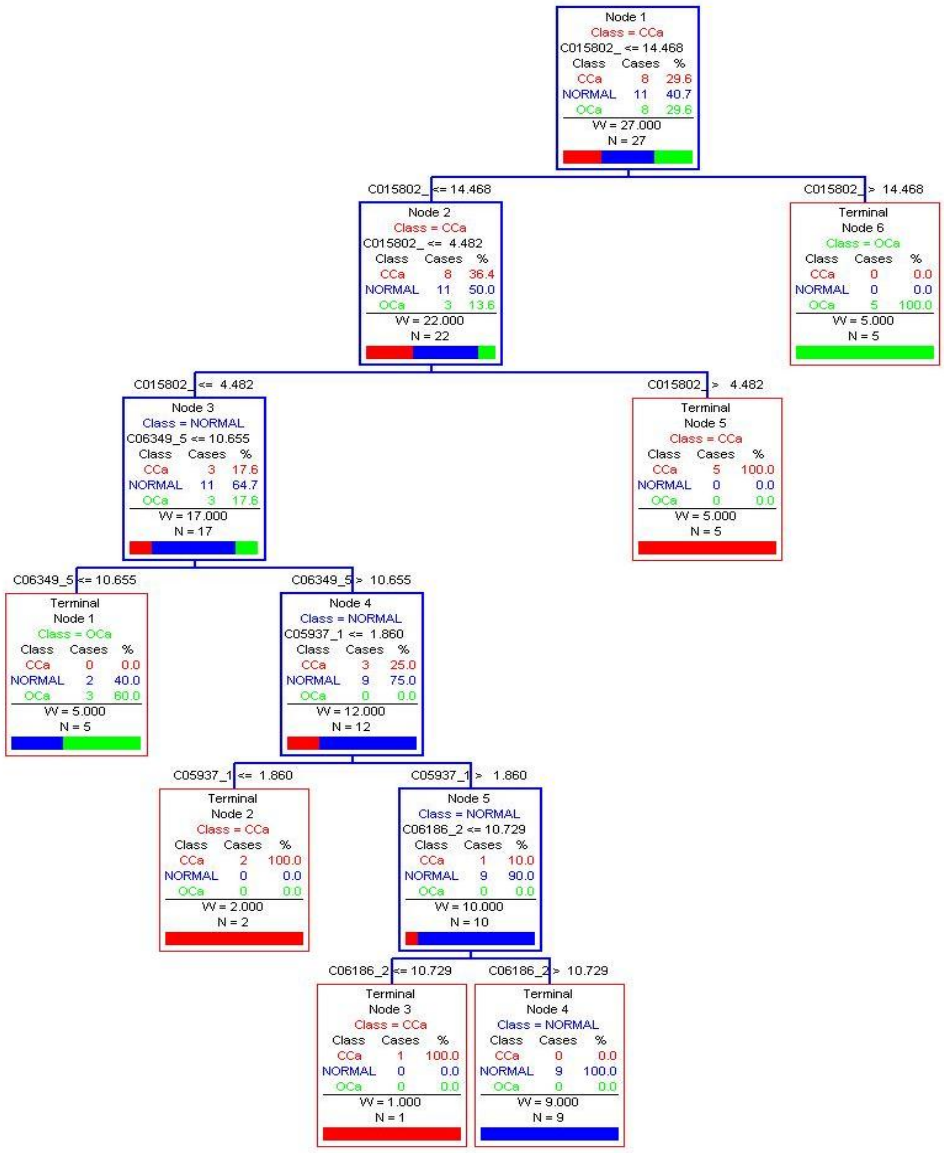


Figure 12: The decision tree of samples upon separation of protein by ProteinChip® Q10 arrays.

A tree structure of the classification model was generated to best classify the OCa patients, CCa patients and control samples using ProteinChip® Q10 Arrays**.** In the hierarchy rectangles, the presence or absence of three SELDI-TOF-MS peaks, with *m/z* value of 5937.1, 6186.2, 6349.5 and 15802.0 served as parent nodes to classify samples in a hierarchical way. Samples were classified into a lower level node until the most correct classification was achieved in the terminal nodes.

(defined by peak *m/z* 6349.5), where two controls and three ovarian cancer samples with peak intensity lower than 10.655 µA were classified into Terminal Node 1. The other 12 samples entered Node 4 (defined by peak *m/z* 5937.1), where two cervical cancer samples with peak intensities of 1.86 µA or less went to Terminal Node 2. At Node 5 (defined by peak *m/z* 6186.2) one cervical cancer sample with peak intensity lower than 10.729 µA entered Terminal Node 3 and the remaining nine samples of control healthy women with peak intensity higher than 10.729 µA were classified to Terminal Node 4. Overall, from 27 samples, the model classified them into nine controls, ten ovarian cancers and eight cervical cancers.

3.6.5 Spectrum of control, OCa and CCa samples

The *p*-value of the protein peaks in the terminal nodes of the classification tree were obtained from the Chipergen Express Data Manager (Table 6). It was calculated base on the peak intensity of the proteins from OCa, CCa and control. The peaks of *m/z* 7528.7 and at *m/z* 8828.8 obtained at the terminal node of the classification tree using ProteinChip® CM10 Arrays were found to be significantly different. Only one protein, which is peak at m/z 15802, was found to be significantly different in the classification tree obtained from the separation of ProteinChip® Q10.

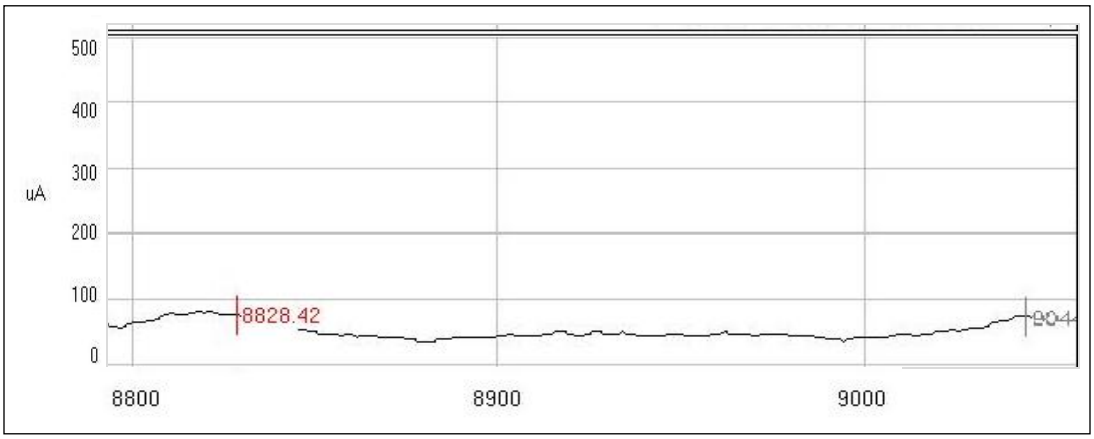
Figure 13 is the representative mass spectrographic profile showing the *m/z* 8828.8 peak which was aberrantly excreted in seven CCa patient with *p*-value 0.03 when compared to both OCa and control samples. Representative of spectra for 7528.78 peak is shown in Figure 14. The abnormally excreted protein was detected in 6 OCa samples with *p*-value less than 0.01 when compared to both CCa patients and control samples. Figure 15 is the representative mass spectrographic profile reveals higher intensity of *m/z* 15802 peaks in

Table 6: Statistical analysis of protein peak intensity from OCa, CCa and control by Chipergen Express Data Manager.

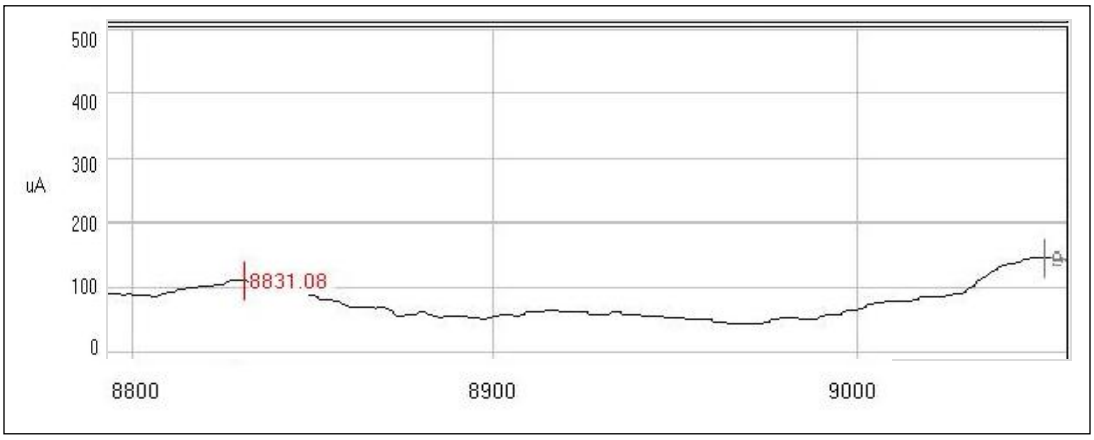
Protein peaks from ProteinChip® CM10	
m/z	*P* value
5393.9	0.70
6176.7	0.18
7528.7	0.01
8828.8	0.03

Ptotein peaks from ProteinChip® Q10	
m/z	*P* value
5937.1	0.11
6186.2	0.11
6349.5	0.18
15 802	0.01

(A) Control



(B) Ovarian cancer patient



(C) Cervical cancer patient

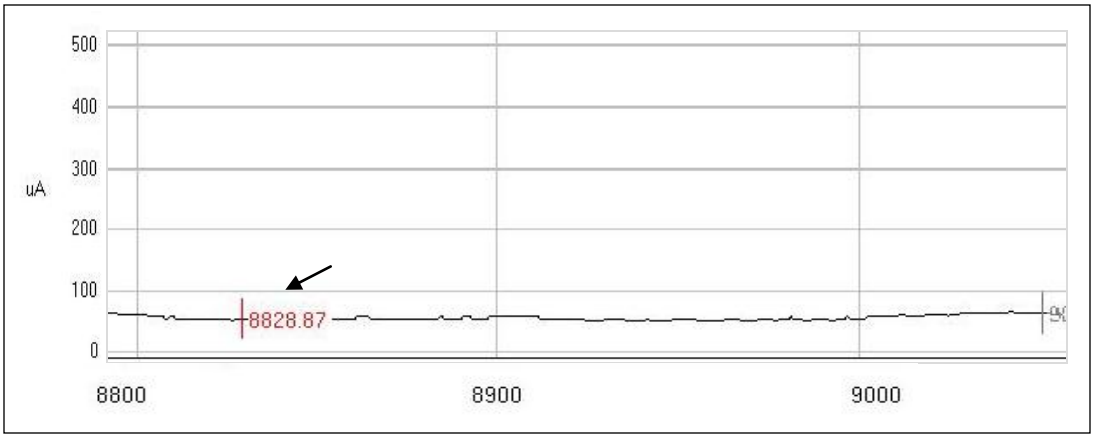
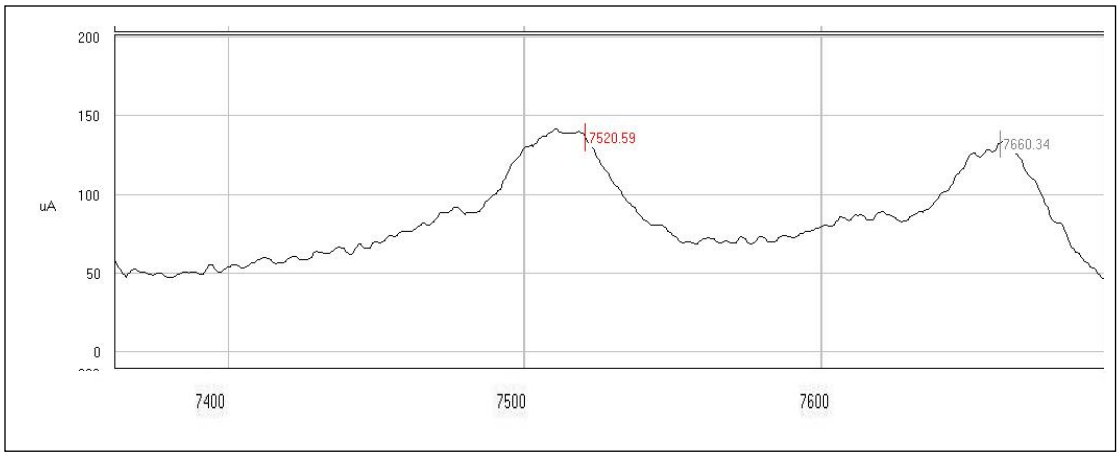
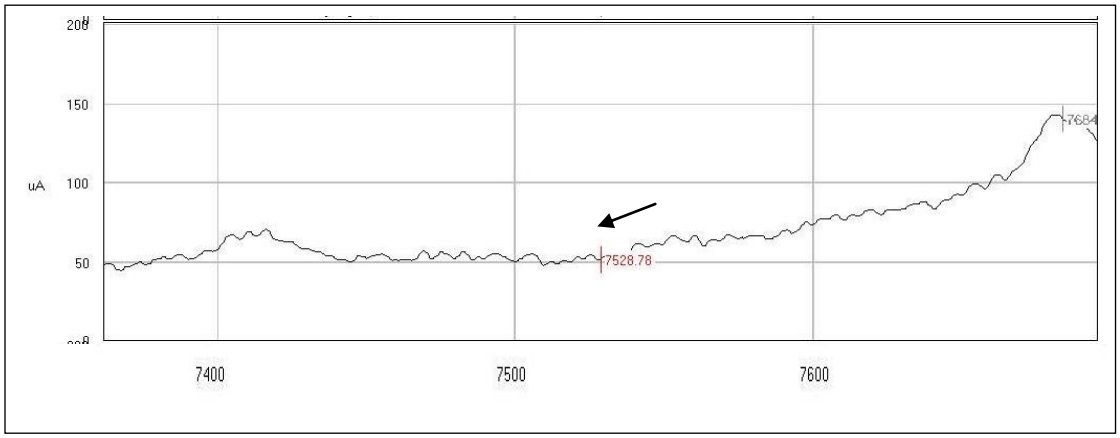


Figure 13: SELDI-TOF-MS spectra showing peak *m/z* 8828.8 of control, OCa and CCa samples using ProteinChip® CM10 array.
Panel A, B and C are the representative mass spectrographic profile showing the *m/z* 8828.8 peak which was aberrantly excreted in seven cervical cancer patient with *p*-value less than 0.05 when compared to both OCa and control samples. Arrow show no peak detected in sample, indicating that less protein is excreted in CCa patients.

(A) Control



(B) Ovarian cancer patient



(C) Cervical cancer patient

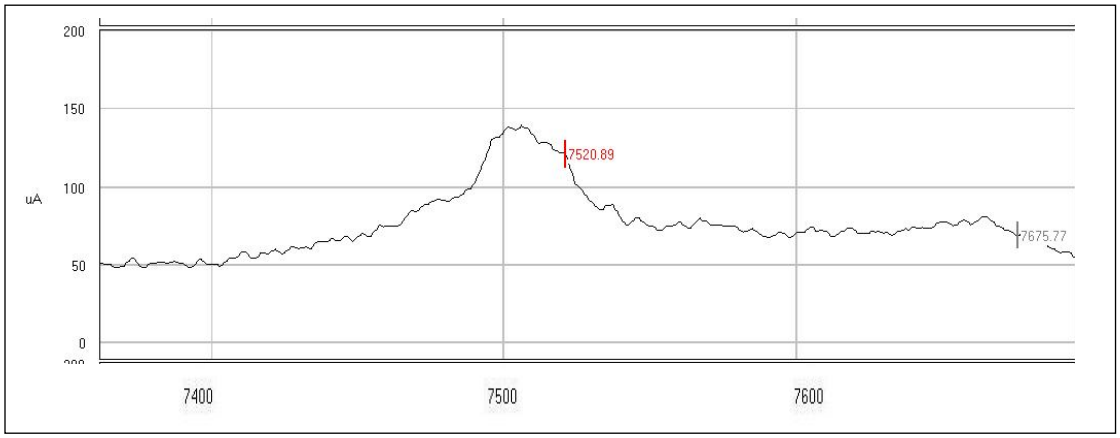
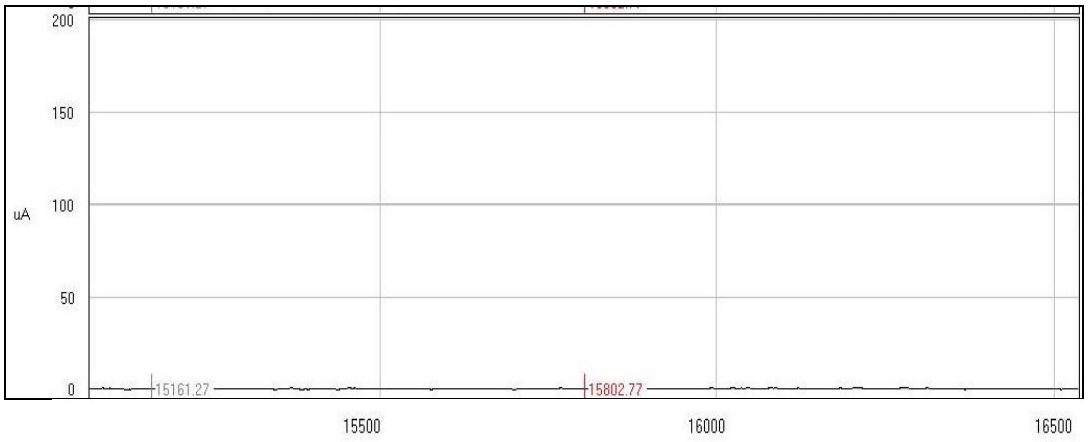


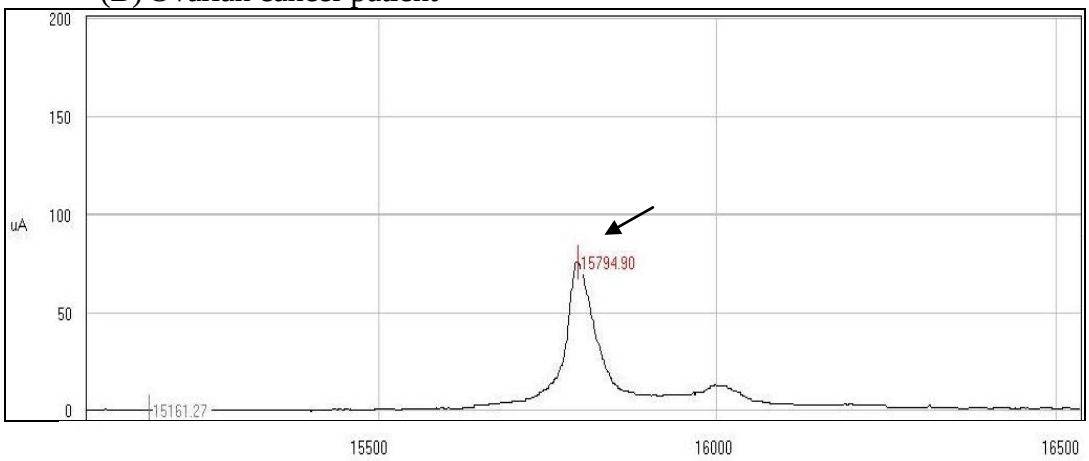
Figure 14: SELDI-TOF-MS spectra showing peak *m/z* 7528.78 of control, OCa and CCa samples using ProteinChip® CM10 array.

Panel A, B and C are the representative mass spectrographic profile showing the *m/z* 7528.78 peak which was aberrantly excreted in six ovarian cancer patient with *p*-value less than 0.05 when compared to both CCa and control samples. Arrow show no peak detected in sample, indicating that less protein is excreted in OCa patients.

(A) Control



(B) Ovarian cancer patient



(C) Cervical cancer patient

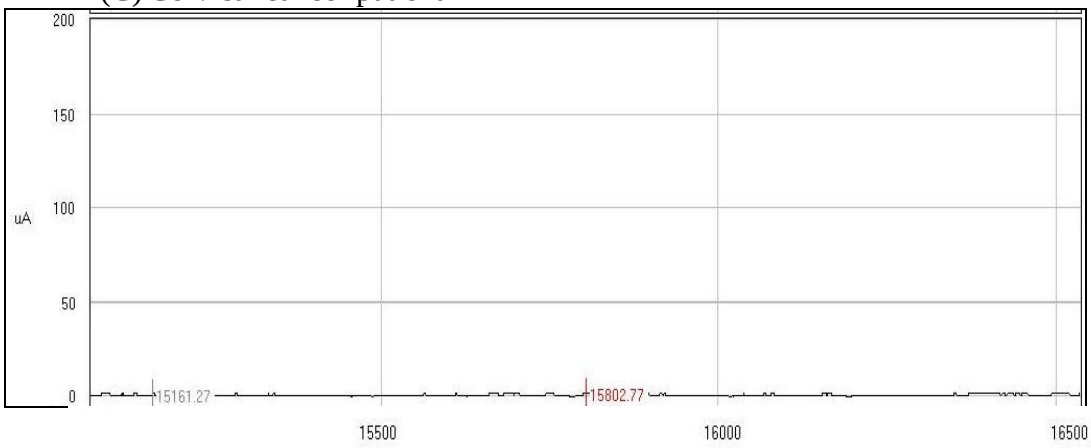


Figure 15: SELDI-TOF-MS spectra showing peak *m/z* 15,802 of control, OCa and CCa samples using ProteinChip® QM10 array.

Panel A, B and C are the representative mass spectrographic profile showing the *m/z* 15802 peak which was aberrantly excreted in five ovarian cancer patient with *p*-value less than 0.05 when compared to both CCa and control samples. Arrow show high peak detected in sample, indicating that the protein is excreted more in OCa patients.

five ovarian cancer patients with *p*-value of 0.01 compared to both cervical cancer patients and control peaks. On average, the peak intensity in ovarian cancer samples were about 30 µA but it was hardly detected in cervical and control samples.

3.7 Sensitivity and specificity of the tree summary report

To validate the sensitivity value obtained from the BPS software, it was recalculated using the formula (Altman *et al.,* 1994) in section 2.8.4.3. The sensitivity value obtained from the software was similar with the value calculated using the formula. The specificity was calculated based on the formula reported by Altman *et al.,* (1994) since the specificity value was not given by the BPS software. Table 7 and Table 8 demonstrate the sensitivity and specificity of the learning set and test set of the diagnostic decision tree of the protein peaks separated by CM10 ProteinChip® array and Q10 ProteinChip® array, respectively.

Referring to Figure 11, the four peaks at *m/z* 5393.9, 6176.7, 7528.7, and 8828.8 were used to generate the classification tree to classify the three cohorts. Based on the tree, 19 out of 27 samples were correctly classified as no-ovarian cancer, where the specificity of the classification method was 100%. However, the sensitivity was only 87.5% since one of the OCa sample was misclassified as cervical cancer. Unlike the CCa samples, the tree manage to classify all the eight cervical cancer thus, achieved the sensitivity of 100%. However, the specificity was only 89.47% since two samples belong to control and OCa was also classified as cervical cancer (Table 7).

The cross validation which generate the test set had correctly classified five out of eight ovarian cancer samples and nine out of nineteen non-ovarian cancer samples. As for the cervical cancer, the test set had correctly classified three out of eight cervical cancer samples and eleven out of 19 no-cervical cancers. These results yielded sensitivity of

62.5% and specificity 47.36% for the ovarian cancer whereas sensitivity of 37.5% and specificity of 57.9% for the cervical cancer test set (Table 7).

Referring to Figure 12, the four peaks at *m/z* 5937.1, 6186.2, 6349.5 and 15802 were used to generate the classification tree to classify the three cohorts. Based on the tree, eight out of 27 samples were classified as ovarian cancer, where the sensitivity of the classification method was 100%. However, the specificity was only 89.5% since two out of the 19 no-ovarian cancer samples were misclassified as ovarian cancer. Interestingly, 100% sensitivity and 100% specificity were achieved by this classification model to correctly classify all the eight cervical cancer and 19 no-cervical cancers samples.

For the test set, four out of eight ovarian samples were correctly classified and five out of 19 true non-ovarian cancer yielded sensitivity of 50% and specificity of 26.3%. As for the test set on cervical cancer patients, one out of eight cervical cancer samples and eight out of 19 non-cervical samples were correctly classified. These result yielded sensitivity of 12.5% and specificity of 42.1% (Table 8). The summary of the prediction result of the diagnostic model of its sensitivity and specificity for ovarian cancer and cervical cancer were shown in Tables 7 and 8.

Table 7: The prediction results of the diagnostic model for ovarian cancer and cervical cancer separated by ProteinChip CM10.

Group	Sample	Cases	Correctly classified samples*	Classification Accuracy (%)
Learning set	Ovarian cancer	8	7	87.5[a]
	No-ovarian-cancer	19	19	100[b]
Test set	Ovarian Cancer	8	5	62.5[a]
	No-ovarian-cancer	19	9	47.4[b]
Learning set	Cervical cancer	8	8	100[a]
	No-cervical-cancer	19	17	89.47[b]
Test set	Cervical Cancer	8	3	37.5[a]
	No-cervical-cancer	19	11	57.9[b]

*Correctly classified: Number of samples that were correctly classified by the BPS

[a] Sensitivity

[b] Specificity

Table 8: The prediction results of the diagnostic model for ovarian cancer and cervical cancer separated by ProteinChip Q10.

Group	Sample	Cases	Correctly classified samples*	Classification Accuracy (%)
Learning set	Ovarian cancer	8	8	100[a]
	No-ovarian-cancer	19	17	89.5[b]
Test set	Ovarian Cancer	8	4	50[a]
	No-ovarian-cancer	19	5	26.3[b]
Learning set	Cervical cancer	8	8	100[a]
	No-cervical-cancer	19	19	100[b]
Test set	Cervical Cancer	8	1	12.5[a]
	No-cervical-cancer	19	8	42.1[b]

*Correctly classified: Number of samples that were correctly classified by the BPS

[a] Sensitivity

[b] Specificity

Chapter 4
DISCUSSIONS

4.1 Proteomic analysis and urinary protein profiles

Studies on urinary proteome using different approaches had been previously demonstrated. For example, through the use of HPLC-ESI-MS technique, several peptides and protein fragments had been identified (Heine *et al.*, 1997). Common analytical methods used in study urinary proteome includes SELDI-TOF-MS (Papale *et al.*, 2007), microarray (Liu *et al.,* 2009) and capillary electrophoresis coupled to mass spectrometry (CE-MS) (Zürbig *et al.,* 2009). However, two main approaches, which are 2-DE and SELDI-TOF, have been used to establish a normal human urinary proteome (Jose *et al.,* 2007) and formed the basis for the choice of the techniques used in this study.

In this present study, 2-DE with MS identification and SELDI-TOF were adopted for the screening of diseased and non-diseased individuals. 2-DE was preferred in this study due to its high resolution capacity, which is very important for proteome analysis. It is also applicable in the studies requiring the quantitative analysis of a differential proteome, for example identifying specific protein features between normal and breast malignant cells (Stein & Zvelebil, 2002).

SELDI-TOF technique on the other hand, is preferred because of its reproducibility and its capacity to permit rapid comprehensive large-scale analysis of individual proteins within complex protein mixtures. By using stationary phase, different sample complexity can be reduced which also allow the detection of lower abundance proteins (Lin *et al.,* 2004). SELDI-TOF allows integral protein profiling from a variety of complex biological materials such as serum, blood, plasma, tissue, urine, saliva and cell lysis products with limited sample preparation. It is more sensitive, rapid and requires smaller amounts of sample compared to other proteomics methods (Ionela, Radu, & Elena, 2010).

4.1.1 Optimization in sample collection and sample preparation

The concern of getting a good urinary proteome map started from the beginning of the whole process especially during the sample collection. In order to ensure that the sample was optimally preserved from bacterial growth throughout the whole project, 20 mM sodium azide was added to the urine upon the sample collection. Although preservative had been added, the contaminant in the urine samples had to be removed. The purpose of centrifugation at 10,000 rpm was to remove contaminant cells and cell walls, microorganism and debris. These contaminants would also interfere with the proteome profile by giving dubious spots with consequences of leading to the wrong analysis. The supernatant was then dialysed in order to reduce the salt concentration which would interfere with isoelectric focusing.

According to Thongboonkerd *et al*., in 2007, overgrown bacteria can lead to the additional spots in 2-DE and the increased amount of total protein. They had systematically evaluated the optimum concentration of preservatives and temperature condition which could delay the bacterial growth. Addition of 10 mM sodium azide or 200 mM boric acid as preservative would delay the bacterial growth up to 48 hours upon storage at RT or 4˚C. They also demonstrated that, by keeping the samples at 4°C, there was no bacterial overgrowth observed in the centrifuged samples in the presence of a preservative at 0.1-10 mM sodium azide and 2-200 mM boric acid.

Another controversial subject was the large inter-subject variation in 2-DE urinary protein profiles which made it difficult to establish a normal human urine 2-DE proteome. In this whole study, the urine samples were analyzed individually in 2-DE analysis except for the Western blot technique. It was important to subject the urine sample individually to the 2-DE instead of pooled samples because any extra protein spot that belongs to the

individual urinary sample could be observed in the individual. By employing the statistical analysis, the extra protein spot would become insignificant.

4.1.2 Urinary proteome map

Figure 4 and 5 were the representative urinary proteome map for healthy control women, ovarian and cervical cancer patients where the protein loaded to the 2-DE analysis were optimized as 300 µg. Being a filtrate of blood, urine also contains protein components similar to that of serum (Barrat and Topham, 2007). Thus, abnormalities that can be detected in the serum could also be detected in the urinary proteome. However, unlike serum sample, the concentration of protein in the urine varies broadly in each healthy individual. The concentration of urinary protein from healthy control women obtained in this study was approximately from 38-70 µg/ml. The amount of protein excreted per day by normal adults is usually given as less than 150 mg per 24 h (Castleman 1974; Diem *et al.*, 1962) and comparable to the range obtain from healthy control in the present study.

As for the ovarian and cervical cancer patients, the concentration of urinary protein was around 259-2091 µg/ml and 156–1918 µg/ml, respectively. The higher concentration of protein in both diseases were expected because elevated plasma protein levels is commonly associated with most pathological condition, and often mirrored in the urine as a result of glomerular filtration of small circulating protein and peptide markers that can pass through the glomerular filter (Barrat and Topham, 2007). However, no research has focused on the association of proteinuria with the two cancers although this was observed in some cancers such as multiple myeloma and bladder cancer (An, 2011). It is important to note that the two cancers had been known to spread to other parts and particularly the bladder which could partly explain the proteinuria in their study. The individual is considered proteinuria when the concentration of the proteins found in the urine is more than 200 µg/ml.

4.1.2.1 Comparison of the proteome map with previously published data

Figure 4 demonstrates a representative urinary proteome profile obtained from a control subject. In the present study, separation of urinary protein samples by 2-DE resulted in highly resolved profiles comprising more than ten clusters of protein spots. Seven protein spot clusters consistently appeared in all the 15 control samples analyzed and there was no apparent difference in the intensity of the spots between the individual urine samples studied.

Protein clusters labelled 1 to 4 in the control urinary proteome in this study were observed in previously published urinary proteome (Anderson *et al.,* 1979; Thongboonkerd *et al.,* 2002; Pieper *et al*., 2004; Thongboonkerd *et al.,* 2005; Laurence *et al.,* 2011). These proteins were commonly observed since the first proteomic study of normal urine performed in 1979 although at that time the term proteomics had not been established.

In the present study, the protein identification was not performed for all the protein spots or clusters on the proteome map since the main aim for this project was to detect and identify the abnormally or aberrantly excreted proteins. However, based on the protein identification using MALDI-TOF-MS in previous studies, the protein clusters labelled 2 and 4 were believed to be zinc alpha-2 glycoprotein and alpha-1 microglobulin respectively (Pieper *et al*., 2004).

Protein cluster labelled 5 in this study were also believed to consist amylase, alpha 1A, amylase alpha 2A and Chaperon containing TCPI, subunit 6A when compared to the study by Thongboonkerd *et al.,* in 2002. This protein cluster was also identified in samples obtained from healthy male and female donors which were fractionated into native proteins higher and lower than 30 kDa using size exclusion chromatography (SEC) column (Pieper *et al*., 2004).

The widely separated cluster 7 consists of albumin fragment, agrin fragment, post-translationally modified of prostaglandin-H2 $_{D}$-isomerase and caspase 10 fragment (Pieper *et al*., 2004). Previously, cluster 7 were claimed to consist immunoglobulins light chains too (Anderson *et al.*, 1979; Edward *et al.*, 1982). The presence of Ig gamma-1 chain C region and Ig gamma-3 chain C region in the urine were proven using LC-MS-MS for protein identification (Pieper *et al*., 2004). However, the positions of these proteins at cluster 7 were doubted since the molecular weights of these proteins reported by Piper *et al.*, (2004) were much smaller than what was suggested by the Swiss-Prot database unless the peptide detected was a fragment of the protein. Protein spot 9 in the present study was believed to be fibrinogen beta chain fragment (Pieper *et al*., 2004).

4.2 Image analysis of 2-DE, protein identification and validation of the identified proteins

The altered level of proteins in the urine of patients with ovarian carcinoma and cervical cancer, relative to the controls was confirmed when their 2-DE urine protein profiles were subjected to image analysis using the Image Master 2D Platinum Software 7.0. Image analysis also confirmed that the levels of the other highly resolved urine protein spot clusters were comparable between the two cohorts.

Figure 6 demonstrated the mean percentage of volume contribution of the four urinary proteins of interest in control subject and patients with ovarian carcinoma. On the other hand, Figure 7 demonstrated the mean percentage of volume contribution of the three urinary proteins of interest in control subjects and patients with cervical cancer. Table 3 demonstrated that most of the proteins namely CD59 and ITIH4 fragment were down regulated in the ovarian carcinoma and cervical cancer but albumin fragment was appeared to be higher in both ovarian and cervical cancer patients when compared to the control.

Although the albumin fragment was appeared 4-fold higher in cervical cancer patients when compared to the ovarian cancer patients, the different was not significant.

The spot clusters of urinary proteins that were aberrantly excreted were then subjected to mass spectrometry and database search for protein identification. Table 4 shows a summary of the data acquired. The database searched identified them as CD59, kininogen-1, inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and albumin. The other set of similar aberrantly excreted urinary proteins samples sent to APAF for comparison purposes, also gave similar identification.

Table 5 demonstrated the list of matched peptide sequences of high confidence identified from MS/MS analysis. In this study, the identification of the proteins was successfully done with the MOWSE protein score greater than 69 which is the value considered significant by the MASCOT database searches. Among the four urinary proteins of interest, ITIH4 and albumin demonstrated large discrepancies between the experimental masses that were estimated based on their mobilities on the 2-DE gels and their theoretically calculated mass. This suggested that the ITIH4 and albumin spots detected in the 2-DE urinary protein profiles were truncated fragments of their native molecules.

In the case of ITIH4 (Q14624), the peptide sequences identified with high confidence from the MS/MS correlated to the C-terminal region of the protein, when they were checked against the Swiss-Prot database. Sequences obtained were those that spanned within the kallikrein-generated 35 kDa fragment region of ITIH4 (amino acids 696-930). However, molecular mass estimation based on its relative mobility in 2-DE gels indicated a larger fragment of approximately 39 kDa.

In the case of KNG1, high-molecular-weight kininogen (HK) and low-molecular-weight kininogen (LK) proteins are the two isoforms produced by alternative mRNA splicing in human (Takagaki *et al.,* 1985; Kitamura *et al.,* 1985). Although in the present

study demonstrated the molecular weight of KNG1 was 64.34 kDa which is the size normally observed in LK (Sainz *et al.,* 2007), the important sequence which differ the HK and LK proteins was not observable.

LK could be distinguished from HK by observing the LK sequence at position 402-427, VSPPHTSMAPAQDEERDSGKEQGHTR in HK which is replaced by SHLRSCEYKGRPPKAGAEPASEREVS in LK. Apart from that, LK protein is shorter than the HK because of the missing sequence at 428-644 during the alternative mRNA splicing (www.uniprot.org). Based on the MALDI-TOF-MS/MS for KNG1 sequence in this study, it could not be determined whether the HK or LK isoform was present since the sequence from 402-427 was not detected by the MS/MS.

Figure 8 in this study showed further confirmation of the altered levels of CD59, kininogen-1 and fragments of ITIH4 and albumin in the urine of patients with ovarian carcinoma relative to those of the control subjects was performed using antisera and a lectin that bind to the respective proteins that were blotted onto membranes. The interactions of proteins demonstrated in pooled control, ovarian and cervical cancer patients validated the identification of proteins obtained from MALDI-TOF-MS/MS. In case of the 19 kDa albumin fragment, interaction appeared to be detected in the pooled urine of patients with ovarian carcinoma and cervical cancer compared to that of the controls, while the inverse was observed for CD59, kininogen-1 and the 39 kDa fragment of ITIH4.

Unlike other proteins which interacted with the commercial antisera, ITIH4 was not detected by the antibody. It is probably due to the loss of epitope recognized by the antibody when the protein was fragmented. This is supported by the fact that the antibody could detect the 103 kDa ITIH4 but not the 39 kDa fragment. To overcome the problem in this study, CGB lectin was used to detect the C-terminal O-glycosylated ITIH4 fragment

(Mohamed *et al.,* 2008) and the results obtained validates the decrease in the excretion of the ITIH4 fragment as observed in silver stained 2-DE profile.

4.3 Compliment Regulatory Protein CD59

The CD59, which was found to be down regulated in the urine of OCa and CCa patients, is a membrane glycoprotein that functions to inhibit membrane attack complex (MAC) by binding to C5b-8 complex and thus inhibiting the recruitment of C9 (Lachman, 1991). Thus, CD59 plays an important role in inhibiting the lysis of normal self cell by complements during the process of inflammations. CD59 is anchored to the cell membrane through glycophosphotidylinositol which is linked to the C-terminal residue Asn-77 (Meri *et al.,* 1990). The soluble form of CD59 can normally be found in urine with the concentration of 3.7 µg/ml (Lehto *et al.,* 1995). However, the soluble form could hardly be detected in the serum or plasma but through addition of detergent to the serum or plasma, the concentration of 33-119 ng/ml CD59 could be detected using ELISA assay (Landi *et al.,* 2003). This might explain why the association of serum CD59 and cancer has not been reported although the differential expression of CD59 on tumours (Madjd *et al.,* 2003; Watson *et al.,* 2006) and in urine (Week *et al.,* 2008; Kreunin *et al.,* 2007) was documented. Since the CD59 was hardly being detected in the serum, it was an advantage to use urine as a sample for the detection of the CD59 protein.

Like the normal cells, cancer cell lines of the breast (Ellison *et al.,* 2007), lung (Zhao *et al.,* 2009) and ovary (Bjorge *et al.,* 2005) which express CD59 were shown to resist the lysis by complements when the cells were exposed to specific monoclonal antibody. Therefore, it is possible that the expression of CD59 and other complement regulatory proteins on cancer cells can inhibit the elimination of the cancer cells through

classical complement pathway or antibody-dependent cell-mediated cytotoxicity (ADCC) and allow the cells to develop into more malignant cells. Although the *in vitro* study seems to suggest the expression would be beneficial to the cancer cells, the analysis on the colorectal cancer cells however, showed the higher expression of CD59 in earlier stages of tumour and then the expression decreases in poorly differentiated and metastasised cells (Watson *et al.,* 2006). The correlation of the expression in the urine samples and the progress of the diseases are yet to be determined.

Although similar reduced excretion of the protein had previously been reported in the urine of patients with bladder cancer (Kreunin *et al.,* 2007) and pancreatic ductal adenocarcinoma (Week *et al.,* 2008), the decrease in expression of CD59 in urine of patients with cervical cancer and ovarian carcinoma was never reported previously. The reason for the low levels of CD59 in the urine of cancer patients is not understood. One possibility is that since the turnover of cancer cells bearing CD59 is low as they are generally "immortal", less of the cell surface molecules are being solubilized and excreted in the urine. However, this remains to be further proven.

4.4 Kininogen-1

Like CD59, kininogen-1 was also detectable in the urine of healthy individuals. Previous studies performed on serum and plasma samples have shown that the expression of kininogen-1 was significantly reduced in patients with gastrointestinal cancer (Roeise *et al.*, 1990), breast cancer (Doustjalali *et al.,* 2004) and two different types of cervical cancer (Abdul-Rahman *et al.,* 2007). Since kininogen-1 is known for its antiangiogenic properties and inhibitory action on the proliferation of endothelial cells (Liu *et al.,* 2008), its lowered expression in serum or plasma of the cancer patients was believed to have contributed to the survival of the cancer cells (Abdul-Rahman *et al.,* 2007).

In view of these previous reports, it was not surprising to find similar reduced levels of kininogen-1 in the urine of patients with ovarian carcinoma (figure 6). However, the aberrant kininogen-1 expression is apparently not cancer-specific since decreased levels of the protein had previously been reported in the urine of patients with chronic pancreatitis (Weeks *et al.,* 2008), interstitial cystitis (Canter *et al.,* 2008) and IgA nephropathy (Rocchetti *et al.,* 2008), although the cause for the altered levels of kininogen-1 in these diseases may have been different.

The role of KNG1 either is inducing angiogenesis or apoptosis, which leads to the inducing or inhibiting cancer, remains unclear since the molecular mechanisms involved both process are extremely complex (Carmeliet e*t al.,* 2001; Eliceiri *et al.,* 2001; Folkman *et al.,* 1995). However, it was shown previously that Brown Norway-Katholiek (BN-Ka) rats, which lack of kininogen due to a single mutation, could not generate bradykinin and display decreased in angiogenesis (Hayashi *et al.,* 2002). Interestingly, administration of a bradykinin analog to BN-Ka was shown to promote angiogenesis associated to the upregulation of the Vascular Endothelial Growth Factor (Ku *et al.,* 1993, Hayashi *et al.,* 2002). Another study directly displayed that daily injection of KNG1 to BN-Ka rats enhance angiogenesis (Ikeda et al., 2004). It is possible that the decrease in kininogen-1 excretion in urine is due to the increase of its usage for cancer survival.

Although KNG1 was also less excreted in the urine of cervical cancer patients, it was statistically not significant (Table 3). Previously, the KNG1 in the serum of squamous cell cervical carcinoma and cervical adenocarcinoma were found as 0.01 and 0.3 fold decrease in expression when compared to the control (Abdul-Rahman *et. al.,* 2007). This slight different found in the serum might explain the reason why the analysis in the urine was not significant. Thus, a higher number of cervical cancer specimens may be required for a statistical analysis in confirming the present data.

4.5 Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4)

ITIH4 which may be involved in acute phase reactions or endopeptidase inhibitory functions, were detected in low levels in patient's urine of ovarian carcinoma and cervical cancer as shown in figure 6 and figure 7. The estimated molecular mass of the urine ITIH4 fragment indicated that it was slightly larger than its reported 35 kDa serum counterpart in the ovarian carcinoma patients (Mohamed *et al.,* 2008). Detection of the different sizes of ITIH4 fragments was not surprising as previous study using SELDI-TOF-MS have demonstrated that ITIH4 was extensively processed within its proline-rich region in the human serum. In different diseases including ovarian carcinoma, different fragments were shown to be proteolytically generated (Song *et al.,* 2006). It was confirmed that the sequence found in the urine samples was similar with the sequence of 35 kDa ITIH4 based on the MALDI-TOF-MS analysis.

While the present study demonstrated the reduced levels of the 39 kDa ITIH4, fragment in the urine of patients with ovarian carcinoma and cervical cancer, the previous data showed the up-regulated levels of a 35 kDa ITIH4 fragment in the serum samples of the patients (Mohamed *et al.,* 2008). This inverse relationship and the difference in the molecular masses of the ITIH4 fragments detected in the respective samples suggest presence of a selective glomerular filtration mechanism that retained the 35 kDa fragment in the blood but allowed its 39 kDa counterpart to be excreted in the urine. The 39 kDa ITIH4 in the urine can be further validated and confirmed by doing the amino acid sequencing in order to see the actual location of the cleavage.

The precise reason for the reduced levels of the ITIH4 fragment in the urine of the patients is not understood. However, ITIH2, ITIH3, ITIH4 and ITIH5 with exception of ITIH1 were found to be downregulated in a variety of human solid tumours investigated in the differential gene expression of the ITIH gene family (Hamm *et al.,* 2008). ITIH2 was

previously studied profoundly and associated to the breast cancer (Hamm *et al.,* 2008), while ITIH4 was found to be less excreted in ovarian and cervical cancer patients in the present study. ITIH4 gene expression was also found to be down regulated in ovarian cancer tissue previously suggesting that certain ITIH protein expression might be specific towards certain cancer diseases.

4.6 Albumin

Based on their resolved locations in the 2-DE gels and MS/MS derived sequences, the enhanced albumin spots detected in the urine of ovarian carcinoma and cervical cancer patients in this study (figure 6 and 7) appeared to be an albumin fragment with the amino acid sequence positions 118 to 281, and with an approximate molecular mass of 19 kDa as shown in Table 5. The human urine is known to contain low levels of albumin fragments, with some polypeptides containing discontinuous sequences joined by unknown crosslinks (Hortin *et al.,* 2008).

Up until now, there are several groups suggesting the potential of albumin as a cancer biomarker. Mider *et al.* in 1984 was the first to report that albumin might be involved in tumour nutrition by concluding that tumour acts as a nitrogen trap after comparing the protein metabolism in normal and tumour tissue. Later, it was demonstrated that albumin entered the tumour cells by pinocytosis (Ryser *et al.,* 1963).

A group of researchers had extensively studied the involvement of albumin by systematically demonstrating that the tumour is the major site of albumin catabolism (Sinn *et al.,* 1990; Schilling *et al.,* 1992). Furthermore, Roger *et al.,* (1989) showed that the residualizing radiolabeled albumin uptake increased not only in ovarian tumour-342, but also in other cases such as Walker-256 carcinoma, Novikoff hepatoma, Dunning prostatic and C6 glioma.

The increase in excretion of albumin fragment found in the urine of patients with ovarian and cervical cancer in this study was most probably due to the by products or waste from the albumin catabolism of the tumour itself. Since the 19 kDa albumin fragment was present only in trace quantities in the urine of the control subjects, it may be used as a complementary urine biomarker to differentiate patients from control.

4.7 SELDI-TOF-MS

SELDI-TOF-MS is becoming one of the most promising tools for the discovery of novel clinical biomarker with the advance and expanding of various types of ProteinChip® surfaces that selectively capture specific subgroups of proteins. This is a unique approach in which peptides are captured on the target itself by virtue of their chromatographic properties. Beside of a good sample preparation and handling technique, the key for a successful analysis in SELDI-TOF-MS is also dependent on the ProteinChip® used. There are many chips arrays manufactured which are specific for hydrophobic, hydrophilic, anionic and cationic proteins. While 2-DE is specifically used to study high molecular weight of proteins, SELDI-TOF-MS technique is more sensitive towards low molecular weight proteins. Thus, SELDI-TOF-MS technique was used as a complimentary method to further analyse the aberrantly expressed proteins in both ovarian carcinoma and cervical cancer when compared to the control.

4.7.1 Optimization of the sample preparation

Originally, in order to obtain optimum low MW protein which is less than 10 kDa, the samples were prepared as described in section 2.3.1 with an exception of dialysis step where the samples was filtered using two milimicron filtration. However, the obscurity

arises when applied to the SELDI-TOF-MS. No peaks were observed although the laser energy had been increased up to 3000 nJ. Although several repetitions had been done, the unpromising result was obtained. One possibility is that, filtration followed by centrifuged and freeze drying did not only concentrate the protein but also the salt. High concentration of salt might have interfered with the binding of the proteins towards the chips although the centrifuged samples and filtration did eliminate the big agregates.

When the urine samples was centrifuged or filtered using two milimicron filtration but not concentrated, only peak intensity up to 60 µA for proteins which was less than 5000 Da were observed, which was dissimilar to that of Roelofen *et al.* (2007) which managed to get 5000 Da with the same protocol. Papale *et al.*, (2007) had demonstrated a better spectrum of the urine supernatant but they had to use higher laser intensity of up to 15000 nJ. However, to increase the laser shoot was not recommended because the used of high laser shoot intensity would shorten its life time and affect the efficiency of the laser shoot when it has to be used frequently. Therefore, dialysed samples were preferable to be used in this study because the removal of the salt gave a better spectrum and more cost effective for a longer use of the analyser.

4.7.2 ProteinChip® CM10 and Q10

In this present investigation, ProteinChip® CM10 and Q10 were chosen for the screening of aberrantly expressed urinary proteins from patients with ovarian and cervical cancer when compared to the control individuals. Evaluations of using various chips with different matrix were not done in this study due to the fact that each type of chips was expensive and not reliable to be tested out. However, based on the previous study, high yield of protein recovery was ensured when tested with CM10 chip in the presence of either

SPA or/and CHCA (Roelofen *et al.,* 2007), as compared to IMAC, NP-20, H-50 and H4 chip arrays.

SPA was found to be the best matrix for Q10, H-50 and IMAC-CU and it would resolve the low molecular weight mass range of 10000 *m/z* for CM10, when compared to CHCA (Papale *et al.,* 2007). Figure 4 showed that most of the low molecular weight proteins were at the acidic side of the 2-DE gel and thus, it was favourable to choose the ProteinChip® Q10 with SPA as the matrix solution. However, in order to ascertain the capture of almost all proteins including the basic proteins, which might not have appeared on the proteome map, ProteinChip® CM10 with CHCA as the matrix was used.

4.8 Diagnostic decision tree

4.8.1 Establishment of a diagnostic decision trees from peaks separated by Protein Chip® CM10 array or ProteinChip® Q10 array

After the quality of the decision trees were evaluated (section 3.6.2.1), the final decision trees were obtained as shown in figure 11 and 12. Figure 11 showed that the model correctly classified ten out of the eleven controls, seven out of the eight ovarian cancers and all the eight cervical cancers. The two samples of control and ovarian cancer were classified as cervical cancers. Figure 12 showed that the model correctly classified nine out of the eleven controls, the eight cervical cancers and eight ovarian cancers except two control samples which the model had misclassified them as ovarian cancer. Since the sensitivity and specificity of the classification tree was determined from the correctly classified diseased and non diseased condition, thus the table as shown in Table 7 and 8 were developed by using the generated misclassification rate from the BPS software.

4.8.2. The implication of low sensitivity, specificity of the test set and broadens peaks towards the constructed diagnostic decision trees

By referring to Tables 7 and 8, it was notable that the constructed decision tree was not good enough to separate the diseased and non diseased condition for the test set. The insignificant protein peaks which were used to construct the diagnostic decision trees were believed to contribute to the high misclassification rate when they were applied to the cross validation method. Referring to Table 6, only *m/z* 7528.7, 8828.8 and 15802 were significant. Although low expression of the protein with *m/z* 8828.8 found in cervical cancer and protein with *m/z* 7528.78 found in ovarian cancer might have a potential as a biomarker, it was hard to decide whether the proteins are good enough to be use as biomarker since the poor separation and broadening of the peaks. The broad peaks might due to the overlapping proteins and other interferences such as nucleotides.

Although the classification trees correctly predicted eight cervical cancer patients from the control and ovarian cancer groups, making the sensitivity and specificity of the classification tress as 100% using ProteinChip® Q10 arrays (Table 8), the classification tree was unable to give good classification when cross validation was performed. This most probably due to the peaks that were used for classification such as peaks at *m/z* 5937.1, 6186.2 and 6349.5, were not significantly detected.

Interestingly, protein peak at *m/z* 15802 with a *p*-value less than 0.05 had a potential to be a good biomarker with 100% sensitivity and 89.4% specificity for the learning set. Figure 15 demonstrated that the protein was well separated and the expression was obvious in the ovarian cancer patient's sample. The diagnostic decision tree can be improved by having a bigger sample size or to create another decision tree base on the artificial data. The

artificial data was created by comparing several variables at a time using linear combination splitting base on the equation below (Wu *et al.,* 2006):

Linear combination split $Y= AX1 + BX2 + CX 3$

4.8.3 Spectrum profile generated from separation of ProteinChip® CM10 and CHCA as the matrix

When using the CM10 ProteinChip® and CHCA as the matrix, two distinct proteins were found to be abnormally expressed in both cancers. The protein that was detected to be down regulated only in urine of cervical cancer patients in the present study was revealed in figure 13. The protein with *m/z* of 8828.8 was found to be down-regulated in seven out of eight cervical cancer samples. Similarly, protein with the mass of 8427 Da was found to be down regulated in the squamous cervical cancer cell by using 2-DE method. The mass spectrometry analysis of the protein indicated the protein as chai x, a crystal of the complex structure between the human Rhoa and Rho-binding domain of human Rocki (Zhu *et al.*, 2009). Interestingly, the down regulation of 8828.8 Da protein in the patients with cervical cancer might be the similar protein demonstrated previously since the pH of the chai x protein is 5.29 and correlate well with chip used in the present study. However, this remains to be further proven and the identification of the protein is compulsory to proof the claim.

Figure 14 showed that the protein peak with *m/z* 7528.78 was down-regulated in the urine of ovarian carcinoma patients. Since the degree of down-regulation is very significant (*p*-value 0.01), this protein has a potential to be a biomarker. Further study need to be done to identify the protein. Interestingly, a study done in 1995 had demonstrated that a 7-kDa protein was over expressed in human multidrug-resistant ovarian cancer cells by developing a monoclonal antibody that specifically recognise this protein. It was suggested

that the over expression of the 7-kDa protein was triggered by the consumption of anti-cancer drug (Yang *et al.,* 1995).

Since the patient in the present study have not gone through any drug treatment to suppressed the ovarian cancer progression, the level of this protein may remain low in the blood and less excreted in the urine and thus, hard to be traced. Another study could be done with urine samples from patients with multi-drug resistant ovarian cancer to see whether the excretion of the protein is high in the urine. If the protein observed in the previous and present study were the same protein, this method and the protein could be used as a therapeutic drug monitoring in the future (Jennie *et al*., 2004). However, purification and identification of the protein is needed to be done. It is possible to be done since several investigators have reported the successful purification and identification of other proteins as markers using tryptic peptide mapping (Rai *et al.,* 2002) and amino acid sequencing (Klade *et al.,* 2001).

4.8.4 Spectrum profile generated from separation of Proteinchip® Q 10 and SPA as the matrix

Figure 15 shows aberrantly expressed protein peak with *m/z* 15802 in the patients with ovarian carcinoma. This was found when using the Q10 ProteinChip® and SPA as the matrix. Although the protein was relatively large, it was not detected by 2DE. It is probably due to the low abundance of the protein in the urine which could only be detected by SELDI-TOF-MS.

In the previous study of early detection in ovarian cancer, the protein peak with *m/z* 15867 was found to be elevated in the serum (Kozak *et al*., 2003; Kozak *et al.,* 2005) and identified as beta-hemoglobin protein by using direct mass-spectral profiling (Karpova *et al.,* 2010). The beta-hemoglobin protein is a known protein that involved in oxygen

transport from the lung to the various peripheral tissues (Ianzer *et al.*, 2006). Extensive and established studies had been done on urine samples (Yoshioka *et al.*, 1986; Jia *et al.*, 1996) which had demonstrated the defect in this protein is the cause of beta-thalassemia, beta-thallassemia dominant inclusion body type (Thein *et al.*, 1990) and sickle cell anemia (Finch *et al.*, 1973; Suzuki *et al.*, 1993).

The 15802 Da protein found in urine sample in the present study might be beta-hemoglobin since the separation done in previous study also done on strong anion exchange chips. In this present study, the reason for the high level of 15802 Da in the urine of ovarian cancer patients is not understood. One possibility is that the elevation of beta-hemoglobin was required since more oxygen is required by the cancer cells signifying that the ovarian cancer cell undergo cancer progression.

The decision tree and the distribution of samples had classified nine out of ten control samples with intensities more than 10.72 μA at protein peak with *m/z* 6186 (Figure 12). Although protein peak with *m/z* 6186 was detected to be low in ovarian and cervical cancer patients when compared to control, the difference was not significant. It was reported previously that protein peak at *m/z* 6195 was down-regulated in the serum of ovarian cancer patients by using SELDI-TOF method (Zhang *et al.*, 2006). It is possible that these two proteins are the same protein where the down-regulation could be observed in both urine and serum.

4.9 Future study

The maturation of MS technologies has given clinical scientist a powerful analytical tool to study human diseases (Aebersold and Mann, 2003). The three aberrantly expressed protein peaks found in this study using the SELDI-TOF-MS approach requires to be optimized in the gel-based method. Although the identifications of the aberrantly expressed

proteins were not determined in this study, the SELDI-TOF-MS technique gave the suggestion of the pH and consideration of acrylamide gel percentage to be used in investigating and identified the proteins of interest. Thus, these proteins can be extracted out by the gel based method and subjected to MALDI-TOF-MS for identification. Another way of identification the proteins derived from the SELDI-TOF-MS is by using columns to extract the protein of interest and immediately subjected them to LC-MS (Pieper *et al.*, 2004) or MALDI-TOF-MS for identification. In this case, extraction using column is preferable since the 2-DE technique done for screening had limitation and did not demonstrate the existence of certain abnormal proteins of interest that can be detected with other method such as SELDI-TOF-MS.

Besides validating these proteins with Western blot technique, further evaluation can be done with larger number of samples and much simpler method using the ELISA technique in order to reassess the abnormality of these protein expressions in a bigger population. Therefore, with more concrete validation and investigation done towards these proteins, it is not impossible to apply the proteins as a biomarker by using the ELISA technique as a clinical practice purposes in the future for an early diagnosis of the OCa and CCa cancers.

4.10 General Conclusion

Taken together, the experiments presented here show that ovarian carcinoma and cervical cancer causes changes in the urinary proteome map that can be used to discriminate between non-cancer control and patients with an early stage of the diseases. Although ovarian carcinoma and cervical cancer shared similar pattern of protein excretion in the urine, which were CD59, ITIH4 and albumin fragment, less excretion of kininogen-1 was found only in patients with ovarian carcinoma could differentiate between the two

diseases (Pang *et al.,* 2010). Complimentary method using SELDI-TOF-MS had facilitated and lead to further findings in searching of potential biomarkes by demonstrating one protein peak with relative molecular mass of *m/z*, 15802 found only in urine of ovarian carcinoma patients. Thus, presenting the combine analysis of 2-DE with MS identification and SELDI-TOF might improve both sensitivity and specificity for early disease diagnosis.

It would be insightful to collect urine from larger cohorts of non-cancer control and diseased patients and to analyse the peptidome and proteome in depth to fully assess the diagnostic potential of these proteomic changes. If the preliminary figures for sensitivity and specificity can be improved, it is conceivable that proteomic analysis of urine might permit prioritisation of investigation between low probabilities of having tumours and to those with higher risk in the future.

APPENDIX

Applied Biosystems

Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	suhana	Database	Sprot
Analysis Name	100609 sample 2	Creation Date	06/10/2009 18:32:25
Reported By	06/11/2009 14:28:27 - 4800	Last Modified	06/10/2009 19:56:51
MS Acq. : Proc. Methods	Training\MS Reflector Positive : Training\MS Reflector Processing Default		
Interpretation Method	Training\Interpretation 20 precursors		

Gel Idx/Pos	53/C2	**Instr./Gel Origin**	AK272/jaime suhana	**Process Status**	Analysis Succeeded
Plate [#] Name	[1] suhana	**Instrument Sample Name**		**Spectra**	21

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
1	CD59 glycoprotein precursor (Membrane attack complex inhibition factor) (MACIF) (MAC-inhibitory pro	CD59_HUMAN	160	100	145	100

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
1539.6646	1539.5757	-0.0889	-58	67	78	FEHCNFNDVTTR	85	100	Carbamidomethyl (C)[4]		Mascot
1539.6646	1539.5757	-0.0889	-58	67	78	FEHCNFNDVTTR			Carbamidomethyl (C)[4]		Mascot
1648.7458	1648.6509	-0.0949	-58	79	90	LRENELTYYCCK	61	99.774	Carbamidomethyl (C)[10,11]		Mascot
1648.7458	1648.6509	-0.0949	-58	79	90	LRENELTYYCCK			Carbamidomethyl (C)[10,11]		Mascot

2	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair	K2C1_HUMAN	141	100	126	100

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
1179.6005	1179.5308	-0.0697	-59	377	386	YEELQITAGR	57	99.469			Mascot
1179.6005	1179.5308	-0.0697	-59	377	386	YEELQITAGR					Mascot
1475.7489	1475.6858	-0.0631	-43	212	223	WELLQQVDTSTR					Mascot
1475.7853	1475.6858	-0.0995	-67	200	211	FLEQQNQVLQTK	50	97.257			Mascot
2383.9517	2383.8066	-0.1451	-61	519	549	GGGGGGYGSGGSSYGS GGGSYGSGGGGGGGR	20	0			Mascot
2383.9517	2383.8066	-0.1451	-61	519	549	GGGGGGYGSGGSSYGS					Mascot

Mascot Search Results

Protein View

Match to: **CD59_HUMAN** Score: **160** Expect: **2.6e-011**
CD59 glycoprotein precursor (Membrane attack complex inhibition factor) (MACIF) (MAC-inhibitory pro

Nominal mass (M_r): **14168**; Calculated pI value: **6.02**
NCBI BLAST search of CD59_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: **18%**

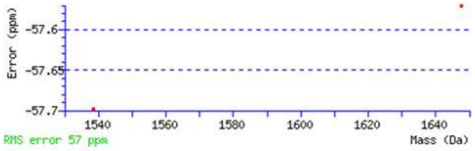
Matched peptides shown in **Bold Red**

```
     1 MGIQGGSVLF GLLLVLAVFC HSGHSLQCYN CPNPTADCKT AVNCSSDFDA
    51 CLITKAGLQV YNKCWKFEHC NFNDVTTRLR ENELTYYCCK KDLCNFNEQL
   101 ENGGTSLSEK TVLLLVTPFL AAAWSLHP
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
67 - 78	1539.58	1538.57	1538.66	-0.09	0	K.FEHCNFNDVTTR.L	Carbamidomethyl (C) (Ions score 85)
67 - 78	1539.58	1538.57	1538.66	-0.09	0	K.FEHCNFNDVTTR.L	Carbamidomethyl (C) (No match)
79 - 90	1648.65	1647.64	1647.74	-0.09	1	R.LRENELTYYCCK.K	2 Carbamidomethyl (C) (Ions score 61)
79 - 90	1648.65	1647.64	1647.74	-0.09	1	R.LRENELTYYCCK.K	2 Carbamidomethyl (C) (No match)




```
ID   CD59_HUMAN              Reviewed;         128 AA.
AC   P13987;
DT   01-JAN-1990, integrated into UniProtKB/Swiss-Prot.
DT   01-JAN-1990, sequence version 1.
DT   20-MAR-2007, entry version 90.
DE   CD59 glycoprotein precursor (Membrane attack complex inhibition
DE   factor) (MACIF) (MAC-inhibitory protein) (MAC-IP) (Protectin) (MEM43
DE   antigen) (Membrane inhibitor of reactive lysis) (MIRL) (20 kDa
DE   homologous restriction factor) (HRF-20) (HRF20) (1F5 antigen).
GN   Name=CD59;
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
OC   Catarrhini; Hominidae; Homo.
OX   NCBI_TaxID=9606;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [MRNA].
RC   TISSUE=T-cell;
RX   MEDLINE=89361238; PubMed=2475570; DOI=10.1084/jem.170.3.637;
RA   Davies A., Simmons D.L., Hale G., Harrison R.A., Tighe H.,
RA   Lachmann P.J., Waldmann H.;
RT   "CD59, an LY-6-like protein expressed in human lymphoid cells,
RT   regulates the action of the complement membrane attack complex on
RT   homologous cells.";
RL   J. Exp. Med. 170:637-654(1989).
RN   [2]
RP   NUCLEOTIDE SEQUENCE [MRNA].
RX   MEDLINE=90168959; PubMed=1689664;
RA   Philbrick W.M., Palfree R.G.E., Roger G.E., Maher S.E., Bridgett M.M.,
RA   Sirlin S., Bothwell A.L.M.;
RT   "The CD59 antigen is a structural homologue of murine Ly-6 antigens
RT   but lacks interferon inducibility.";
RL   Eur. J. Immunol. 20:87-92(1990).
RN   [3]
RP   NUCLEOTIDE SEQUENCE [MRNA].
RX   MEDLINE=89350983; PubMed=2475111; DOI=10.1016/0006-291X(89)90852-8;
RA   Okada H., Nagami Y., Takahashi K., Okada N., Hideshima T.,
RA   Takizawa H., Kondo J.;
RT   "20 KDa homologous restriction factor of complement resembles T cell
RT   activating protein.";
RL   Biochem. Biophys. Res. Commun. 162:1553-1559(1989).
RN   [4]
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Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	suhana	Database	Sprot
Analysis Name	100609 sample 2	Creation Date	06/10/2009 18:32:25
Reported By	06/11/2009 14:29:02 - 4800	Last Modified	06/10/2009 19:56:51

MS Acq. : Proc. Methods: Training\MS Reflector Positive : Training\MS Reflector Processing Default

Interpretation Method: Training\Interpretation 20 precursors

Gel Idx/Pos	175/H2	**Instr./Gel Origin**	AK272/jaime suhana	**Process Status**	Analysis Succeeded
Plate [#] Name	[1] suhana	**Instrument Sample Name**		**Spectra**	8

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
1	Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4) (Inter-alpha-inhibitor	ITIH4_HUMAN	120	100	75	99.991

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
842.4406	842.4473	0.0067	8	627	633	YYLQGAK					Mascot
842.4406	842.4473	0.0067	8	627	633	YYLQGAK					Mascot
996.5771	996.5384	-0.0387	-39	1	8	MKPPRPVR			Oxidation (M)[1]		Mascot
996.5771	996.5384	-0.0387	-39	1	8	MKPPRPVR			Oxidation (M)[1]		Mascot
1551.824	1551.7336	-0.0904	-58	814	826	WKETLFSVMPGLK	31	0	Oxidation (M)[9]		Mascot
1551.824	1551.7336	-0.0904	-58	814	826	WKETLFSVMPGLK			Oxidation (M)[9]		Mascot
2334.1069	2333.9785	-0.1284	-55	911	930	RLDYQEGPPGVEISCWSVEL	24	0	Carbamidomethyl (C)[15]		Mascot
2334.1069	2333.9785	-0.1284	-55	911	930	RLDYQEGPPGVEISCWSVEL			Carbamidomethyl (C)[15]		Mascot
2415.1787	2415.0437	-0.135	-56	754	775	QGPVNLLSDPEQGVEVTGQYER	19	0			Mascot
2415.1787	2415.0437	-0.135	-56	754	775	QGPVNLLSDPEQGVEVTGQYER					Mascot

2	Type II restriction enzyme SacI (EC 3.1.21.4) (Endonuclease SacI) (R.SacI) - Streptomyces achromoge	T2S1_STRAH	44	0	

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start	End	Sequence	Ion	C. I. %	Modification	Rank	Result Type

Mascot Search Results

Protein View

Match to: **ITIH4_HUMAN** Score: **120** Expect: **2.6e-007**
Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4) (Inter-alpha-inhibitor

Nominal mass (M_r): **103261**; Calculated pI value: **6.51**
NCBI BLAST search of ITIH4_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: **7%**

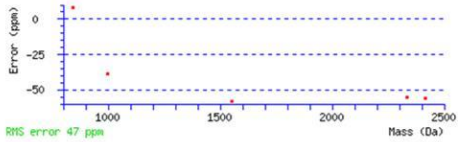
Matched peptides shown in **Bold Red**

```
   1 MKPPRPVRTC SKVLVLLSLL AIHQTTTAEK NGIDIYSLTV DSRVSSRFAH
  51 TVVTSRVVNR ANTVQEATFQ MELPKKAFIT NFSMNIDGMT YPGIIKEKAE
 101 AQAQYSAAVA KGKSAGLVKA TGRNMEQFQV SVSVAPNAKI TFELVYEELL
 151 KRRLGVYELL LKVRPQQLVK HLQMDIHIFE PQGISFLETE STFMTNQLVD
 201 ALTTWQNKTK AHIRFKPTLS QQQKSPEQQE TVLDGNLIIR YDVDRAISGG
 251 SIQIENGYFV HYFAPEGLTT MPKNVVFVID KSGSMSGRKI QQTREALIKI
 301 LDDLSPRDQF NLIVFSTEAT QWRPSLVPAS AENVNKARSF AAGIQALGGT
 351 NINDAMLMAV QLLDSSNQEE RLPEGSVSLI ILLTDGDPTV GETNPRSIQN
 401 NVREAVSGRY SLFCLGFGFD VSYAFLEKLA LDNGGLARRI HEDSDSALQL
 451 QDFYQEVANP LLTAVTFEYP SNAVEEVTQN NFRLLFKGSE MVVAGKLQDR
 501 GPDVLTATVS GKLPTQNITF QTESSVAEQE AEFQSPKYIF HNFMERLWAY
 551 LTIQQLLEQT VSASDADQQA LRNQALNLSL AYSFVTPLTS MVVTKPDDQE
 601 QSQVAEKPME GESRNRNVHS GSTFFKYYLQ GAKIPKPEAS FSPRRGWNRQ
 651 AGAAGSRMNF RPGVLSSRLL GLPGPPDVPD HAAYHPFRRL AILPASAPPA
 701 TSNPDPAVSR VMNIKIEETT MTTQTPAPIQ APSAILPLPG QSVERLCVDP
 751 RHRQGPVNLL SDPEQGVEVT GQYERFKAGF SWIEVTFKNP LVWVHASPEH
 801 VVVTRNRRSS AYKWKETLFS VMPGLKMTMD KTGLLLLSDP DKVTIGLLFW
 851 DGRGEGLRLL LRDTDRFSSH VGGTLGQFYQ EVLWGSPAAS DDGRRTLRVQ
 901 GNDHSATRER RLDYQEGPPG VEISCWSVEL
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
1 - 8	996.54	995.53	995.57	-0.04	0	-.MKPPRPVR.T Oxidation (M) (No match)
1 - 8	996.54	995.53	995.57	-0.04	0	-.MKPPRPVR.T Oxidation (M) (No match)
627 - 633	842.45	841.44	841.43	0.01	0	K.YYLQGAK.I (No match)
627 - 633	842.45	841.44	841.43	0.01	0	K.YYLQGAK.I (No match)
754 - 775	2415.04	2414.04	2414.17	-0.13	0	R.QGPVNLLSDPEQGVEVTGQYER.E (Ions score 19)
754 - 775	2415.04	2414.04	2414.17	-0.13	0	R.QGPVNLLSDPEQGVEVTGQYER.E (No match)
814 - 826	1551.73	1550.73	1550.82	-0.09	1	K.WKETLFSVMPGLK.M Oxidation (M) (Ions score 31)
814 - 826	1551.73	1550.73	1550.82	-0.09	1	K.WKETLFSVMPGLK.M Oxidation (M) (No match)
911 - 930	2333.98	2332.97	2333.10	-0.13	1	R.RLDYQEGPPGVEISCWSVEL.- Carbamidomethyl (C) (Ions score 24)
911 - 930	2333.98	2332.97	2333.10	-0.13	1	R.RLDYQEGPPGVEISCWSVEL.- Carbamidomethyl (C) (No match)

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ID   ITIH4_HUMAN             Reviewed;         930 AA.
AC   Q14624; Q15135; Q9P190; Q9UQ54;
DT   15-JUL-1998, integrated into UniProtKB/Swiss-Prot.
DT   17-OCT-2006, sequence version 3.
DT   20-FEB-2007, entry version 70.
DE   Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy
DE   chain H4) (Inter-alpha-inhibitor heavy chain 4) (Inter-alpha-trypsin
DE   inhibitor family heavy chain-related protein) (IHRP) (Plasma
DE   kallikrein sensitive glycoprotein 120) (PK-120) (GP120) [Contains: 70
DE   kDa inter-alpha-trypsin inhibitor heavy chain H4; 35 kDa inter-alpha-
DE   trypsin inhibitor heavy chain H4].
GN   Name=ITIH4; Synonyms=IHRP, ITIHL1, PK120; ORFNames=PRO1851;
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
OC   Catarrhini; Hominidae; Homo.
OX   NCBI_TaxID=9606;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1), AND PARTIAL PROTEIN SEQUENCE.
RC   TISSUE=Liver;
RX   MEDLINE=95293915; PubMed=7775381;
RA   Saguchi K., Tobe T., Hashimoto K., Sano Y., Nakano Y., Miura N.-H.,
RA   Tomita M.;
RT   "Cloning and characterization of cDNA for inter-alpha-trypsin
RT   inhibitor family heavy chain-related protein (IHRP), a novel human
```

Gel Idx/Pos	76/D1	Instr./Gel Origin	AK272/jaime suhana	Process Status	Analysis Succeeded
Plate [#] Name	[1] suhana	Instrument Sample Name		Spectra	7

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
1	Kininogen-1 precursor (Alpha-2-thiol proteinase inhibitor) [Contains: Kininogen-1 heavy chain; Brad	KNG1_HUMAN	193	100	168	100

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
1003.5683	1003.5428	-0.0255	-25	188	196	QVVAGLNFR	54	98.959			Mascot
1003.5683	1003.5428	-0.0255	-25	188	196	QVVAGLNFR					Mascot
1030.5356	1030.5117	-0.0239	-23	317	324	YFIDFVAR	45	92.013			Mascot
1030.5356	1030.5117	-0.0239	-23	317	324	YFIDFVAR					Mascot
1158.6306	1158.6047	-0.0259	-22	316	324	KYFIDFVAR	69	99.966			Mascot
1158.6306	1158.6047	-0.0259	-22	316	324	KYFIDFVAR					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
2	Trypsin precursor (EC 3.4.21.4) - Sus scrofa (Pig)	TRYP_PIG	65	91.73	56	99.371

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
842.5094	842.4828	-0.0266	-32	108	115	VATVSLPR	56	99.371			Mascot
842.5094	842.4828	-0.0266	-32	108	115	VATVSLPR					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
3	Lecithin retinol acyltransferase (EC 2.3.1.135) (Phosphatidylcholine--retinol O-acyltransferase) -	LRAT_RAT	62	84.601	28	0

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
842.5168	842.4828	-0.034	-40	97	104	LLPGVICK					Mascot
842.5168	842.4828	-0.034	-40	97	104	LLPGVICK					Mascot
1030.5527	1030.5117	-0.041	-40	135	143	SLLNEEVAR	12	0			Mascot
1030.5527	1030.5117	-0.041	-40	135	143	SLLNEEVAR					Mascot
1158.6477	1158.6047	-0.043	-37	134	143	KSLLNEEVAR	16	0			Mascot
1158.6477	1158.6047	-0.043	-37	134	143	KSLLNEEVAR					Mascot

Rank	Protein Name	Accession No.	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
4	Lecithin retinol acyltransferase (EC 2.3.1.135) (Phosphatidylcholine--retinol O-acyltransferase) (P	LRAT_MOUSE	48	0	28	0

Mascot Search Results

Protein View

Match to: **ALBU_HUMAN** Score: **161**
Serum albumin precursor - Homo sapiens (Human)

Nominal mass (M_r): **69321**; Calculated pI value: **5.92**
NCBI BLAST search of ALBU_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: **8%**

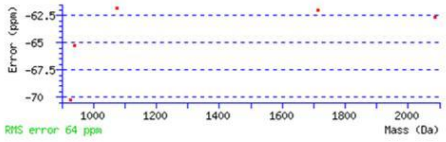
Matched peptides shown in **Bold Red**

```
     1 MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA
    51 FAQYLQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
   101 VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA
   151 FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
   201 CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV ARLSQRFPKA
   251 EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
   301 ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
   351 LGMFLYEYAR RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE
   401 FKPLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV
   451 SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV LNQLCVLHEK TPVSDRVTKC
   501 CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
   551 TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
   601 AASQAALGL
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
118 - 130	1714.69	1713.68	1713.79	-0.11	1	**K.QEPERNECFLQHK.D** Carbamidomethyl (C) (Ions score 30)
131 - 138	940.39	939.38	939.44	-0.06	0	**K.DDNPNLPR.L** (Ions score 37)
162 - 168	927.43	926.42	926.49	-0.07	0	**K.YLYEIAR.R** (Ions score 43)
206 - 214	1074.48	1073.47	1073.54	-0.07	1	**K.LDELRDEGK.A** (Ions score 10)
265 - 281	2086.71	2085.70	2085.83	-0.13	0	**K.VHTECCHGDLLECADDR.A** 3 Carbamidomethyl (C) (Ions score 41)

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ID   ALBU_HUMAN             Reviewed;         609 AA.
AC   P02768; O95574; P04277; Q13140; Q68DN5; Q6UXK4; Q86YG0; Q9P157;
AC   Q9P1I7; Q9UHS3; Q9UJZ0;
DT   21-JUL-1986, integrated into UniProtKB/Swiss-Prot.
DT   01-APR-1990, sequence version 2.
DT   20-MAR-2007, entry version 116.
DE   Serum albumin precursor.
GN   Name=ALB;
GN   ORFNames=PRO0903, PRO1708, PRO2044, PRO2619, PRO2675, UNQ696/PRO1341;
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
OC   Catarrhini; Hominidae; Homo.
OX   NCBI_TaxID=9606;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1), AND VARIANT LYS-420.
RX   MEDLINE=82081882; PubMed=6171778; DOI=10.1093/nar/9.22.6103;
RA   Lawn R.M., Adelman J., Bock S.C., Franke A.E., Houck C.M.,
RA   Najarian R.C., Seeburg P.H., Wion K.L.;
RT   "The sequence of human serum albumin cDNA and its expression in E.
RT   coli.";
RL   Nucleic Acids Res. 9:6103-6114(1981).
RN   [2]
RP   NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1), AND VARIANT GLY-121.
RX   MEDLINE=82105994; PubMed=6275391;
RA   Dugaiczyk A., Law S.W., Dennison O.E.;
RT   "Nucleotide sequence and the encoded amino acids of human serum
RT   albumin mRNA.";
RL   Proc. Natl. Acad. Sci. U.S.A. 79:71-75(1982).
RN   [3]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA].
RX   MEDLINE=86196112; PubMed=3009475;
RA   Minghetti P.P., Ruffner D.E., Kuang W.J., Dennison O.E., Hawkins J.W.,
RA   Beattie W.G., Dugaiczyk A.;
RT   "Molecular structure of the human albumin gene is revealed by
RT   nucleotide sequence within q11-22 of chromosome 4.";
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Patients with ovarian carcinoma excrete different altered levels of urine CD59, kininogen-1 and fragments of inter-alpha-trypsin inhibitor heavy chain H4 and albumin

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Abstract

Background: Diagnosis of ovarian carcinoma is in urgent need for new complementary biomarkers for early stage detection. Proteins that are aberrantly excreted in the urine of cancer patients are excellent biomarker candidates for development of new noninvasive protocol for early diagnosis and screening purposes. In the present study, urine samples from patients with ovarian carcinoma were analysed by two-dimensional gel electrophoresis and the profiles generated were compared to those similarly obtained from age-matched cancer negative women.

Results: Significant reduced levels of CD59, kininogen-1 and a 39 kDa fragment of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and enhanced excretion of a 19 kDa fragment of albumin, were detected in the urine of patients with ovarian carcinoma compared to the control subjects. The different altered levels of the proteins were confirmed by Western blotting using antisera and a lectin that bind to the respective proteins.

Conclusion: CD59, kininogen-1 and fragments of ITIH4 and albumin may be used as complementary biomarkers in the development of new noninvasive protocols for diagnosis and screening of ovarian carcinoma.

Background

Ovarian carcinoma is the leading cause of death among gynaecologic malignancy. It is the fourth most common cancer affecting women in Malaysia [1]. Patients with ovarian carcinoma often presented themselves at an advance stage of cancer mainly because of the lack of biomarker for early diagnosis and that the cancer is usually asymptomatic at the early stages [2]. Once the cancer is detected at the advance stage, the five-year survival rate of the patients decreases to 25% even when appropriate treatments were provided [3,4].

The gel-based proteomic analysis provides a convenient method to compare the levels of proteins in bodily fluid samples. In the search for new protein biomarker candidates with clinical diagnostic value, substantial progress has been made in the proteomic analysis of serum samples of patients with different cancers [5-7]. In contrast, fewer studies have been carried out on the urine samples of cancer patients. This is despite that urine is generally a better sample for investigative and screening purposes and that the use of urine protein biomarkers such as albumin and human chorionic gonadotropin for clinical diagnosis has been a long standing practice.

The proteomic analysis of urine offers ample opportunities for clinical translation [8,9]. To date, proteomic experiments that have been conducted on urine were not confined to patients suffering from diseases of the genitourinary system [10] but were also carried out on those with atherosclerosis [11], sleep disorder [12] and cancers of the bladder [13], pancreas [14,15], lung [16] and colon [17]. Proteomic investigation has been performed on urine of patients with ovarian carcinoma but is currently restricted to the low molecular weight peptide analysis using the SELDI-TOF-MS approach [18].

In the present study, urine protein samples from patients with ovarian carcinoma and cancer negative women were subjected to the conventional two-dimensional

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electrophoresis (2-DE) and densitometry analysis. Proteins that were aberrantly excreted by the cancer patients, relative to control subjects, were identified by mass spectrometry and their altered levels in the patients urine were confirmed by Western blotting using antisera and a lectin that bind to the respective proteins.

Results

2-DE profiles of urine proteins

Separation of urine protein samples by 2-DE resulted in highly resolved profiles comprising more than ten clusters of protein spots. Panel A of Figure 1 demonstrates a representative urinary proteome profile obtained from a control subject. Seven protein spot clusters consistently appeared in all the 15 control samples analyzed and there was no apparent difference in the intensity of the spots between the individual urine samples studied. When the gel-based proteomic analysis was performed on urine protein samples from patients with ovarian carcinoma (n = 11), different 2-DE profiles were obtained (Figure 1, panel B). Three protein spot clusters which consistently appeared in the control profile were either not detected or were reduced in intensity in the cancer patients while one protein spot appeared enhanced in a considerable number of the patients' 2-DE gels. The levels of the other protein spot clusters were comparable to those detected in the urinary proteome profiles of the control subjects.

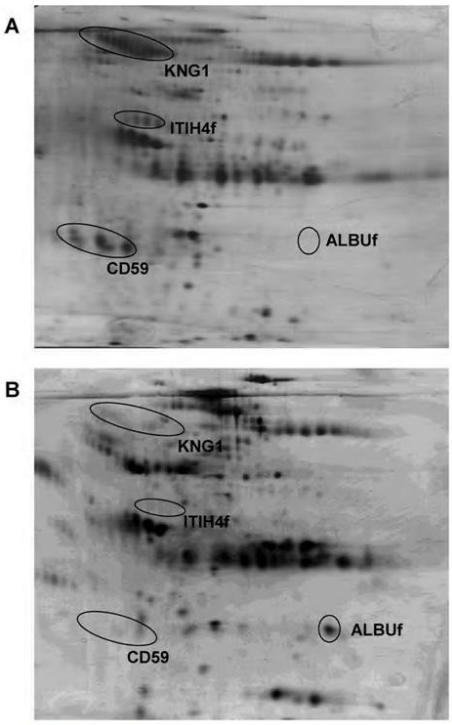

Figure 1 Typical 2-DE urine protein profiles of controls and patients with ovarian cancer. Panels A and B demonstrate the representative 2-DE urine protein profiles of control subjects and ovarian cancer patients, respectively. The aberrantly excreted urine protein spot clusters were marked in circles. KNG1, ITIH4f and ALBUf refer to kininogen-1 and fragments of inter-alpha-trypsin inhibitor heavy chain H4 and albumin, respectively. Acid side of 2-DE gel is to the left and relative molecular mass declines from the top.

Identification of aberrantly excreted urine proteins

Subjecting the spot clusters of urine proteins that were aberrantly excreted to mass spectrometry and database search identified them as CD59, kininogen-1, inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and albumin. Table 1 shows a summary of the data acquired. High probability-based MOWSE scores were obtained for all the urine proteins. Among the four urine proteins of interest, ITIH4 and albumin demonstrated large discrepancies between the experimental masses that were estimated based on their mobilities in the 2-DE gels and their theoretically calculated mass. This suggested that the ITIH4 and albumin spots detected in the 2-DE urinary profiles were truncated fragments of their native molecules.

In the case of ITIH4 (Q14624), the peptide sequences identified with high confidence from the MS/MS correlated to the C-terminal region of the protein, when they were checked against the Swiss-Prot database (Table 2). Sequences obtained were those that spanned within the kallikrein-generated 35 kDa fragment region of ITIH4 (amino acids 696-930). However, molecular mass estimation based on its relative mobility in 2-DE gels indicated a larger fragment of approximately 39 kDa. In case of albumin (P02768), the sequences derived from

Table 1 Mass spectrometric identification of spot clusters from urine protein profiles

Protein	Accession number[#]	Nominal mass (kDa)/*pI*	Mean Experimental mass* (kDa)	MOWSE protein score	Sequence coverage (%)
CD59	P13987	14.168/6.02	19.43	160	18
KNG1	P01042	71.912/6.34	64.34	193	15
ITIH4	Q14624	103.262/ 6.51	38.85	120	7
ALBU	P02768	69.321/5.92	19.45	161	8

Spot ID are as in Fig. 1. KNG1, ITIH4 and ALBU refer to kininogen-1, inter-alpha-trypsin inhibitor heavy chain H4 and albumin, respectively.

[#] Accession numbers are from the Mascot database http://www.matrixscience.com.

*Estimation based on migration in the 2-DE gels relative to protein markers.

Table 2 List of matched peptide sequences of high confidence identified from MS/MS analysis

Peptide sequence	Ion score	Amino acid
39 kDa ITIH4 spots		
R.QGPVNLLSDPEQGVEVTGQYER.E	19	754 - 775
K.WKETLFSVMPGLK.M	31	814 - 826
R.RLDYQEGPPGVEISCWSVEL.-	24	911 - 930
19 kDa albumin spots		
K.QEPERNECFLQHK.D	30	118 - 130
K.DDNPNLPR.L	37	131 - 138
K.YLYEIAR.R	43	162 - 168
K.LDELRDEGK.A	10	206 - 214
K.VHTECCHGDLLECADDR.A	41	265 - 281

Sequences of peptide were checked against ITIH4 (Q14624; 930 amino acids) and albumin (P02768; 585 amino acids) in the Swiss-Prot database.

the MS/MS analysis were confined to amino acids 118 to 281 of the molecule (Table 2).

Image analysis of 2-DE gels

The different altered levels of CD59, kininogen-1 and fragments of ITIH4 and albumin in the urine of patients with ovarian carcinoma, relative to the controls, was confirmed when their 2-DE urine protein profiles were subjected to image analysis using the Image Master 2 D Platinum Software 7.0. Image analysis also confirmed that the levels of the other highly resolved urine protein spot clusters were comparable between cancer patients and controls. Figure 2 demonstrates the mean percentage of volume contribution of the four urine proteins of interest in control subjects and patients with ovarian carcinoma. When taken as overall, the levels of CD59, kininogen-1 and ITIH4 fragment were significantly lower in ovarian carcinoma patients by 3.6-, 2.5- and 1.9-folds, respectively, compared to those excreted by the control subjects. In contrast, the 19 kDa fragment of albumin appeared 274-fold higher in the patients urine (Table 3).

SDS-polyacrylamide gel electrophoresis and Western blotting

Further confirmation of the altered levels of CD59, kininogen-1 and fragments of ITIH4 and albumin in the urine of patients with ovarian carcinoma relative to those of the control subjects was performed using antibodies and a lectin that bind to the respective proteins that were blotted onto membranes. Figure 3 demonstrates the respective interactions of specific antibodies and the CGB lectin with the four proteins of interest in pooled urine samples of patients and control subjects. In case of the 19 kDa albumin fragment, interaction appeared to be detected only in the pooled urine of patients with ovarian carcinoma compared to that of the controls, while the inverse was observed for CD59, kininogen-1 and the 39 kDa fragment of ITIH4.

Discussion

In the present proteomic profiling study, the significant reduced excretion of CD59, kininogen-1 and a 39 kDa fragment of ITIH4, and the enhanced levels of a 19 kDa fragment of albumin were detected in the urine samples of patients with ovarian carcinoma relative to those of the control subjects. Their different altered levels in the urine of ovarian cancer patients were confirmed by Western blotting using antisera and a lectin that bind to the respective proteins. These urinary proteins have potential to be used as complementary molecular indicators for noninvasive diagnoses and/or monitoring of ovarian carcinoma, although this requires further confirmation involving a larger scale clinical investigation.

CD59, a cell surface molecule, functions to inhibit the membrane attack complex of the complement pathway. The soluble form of CD59 is usually found in normal human urine at a concentration of about 3.7 μg/ml. However, it is barely detectable in the blood (between 33-119 ng/ml), and even that only in the presence of detergents [19,20]. To the best of our knowledge, the present study is the first to report the decreased levels of CD59 in the urine of patients with ovarian cancer although similar reduced excretion of the protein had previously been reported in the urine of patients with bladder cancer [13] and pancreatic ductal adenocarcinoma [14]. The reason for the low levels of CD59 in the urine of cancer patients is not understood. One possibility is that since the turnover of cancer cells bearing CD59 is low as they are generally "immortal", less of the cell surface molecules are being solubilized and excreted in the urine. However, this remains to be further proven.

Like CD59, kininogen-1 is also detectable in the urine of healthy individuals. Previous studies performed on serum and plasma samples have shown that the expression of kininogen-1 was significantly reduced in patients with gastrointestinal cancer [21], breast cancer [22] and two different types of cervical cancer [23]. Since kininogen-1 is known for its antiangiogenic properties and inhibitory action on the proliferation of endothelial cells [24], its lowered expression in serum/plasma of the cancer patients was believed to have contributed to the survival of the cancer cells [23]. In view of these previous reports, it was not surprising to find similar reduced levels of kininogen-1 in the urine of patients with ovarian carcinoma in this study. However, the aberrant kininogen-1 expression is apparently not cancer-specific since decreased levels of the protein had previously been reported in the urine of patients with chronic pancreatitis [14], interstitial cystitis [25] and IgA nephropathy

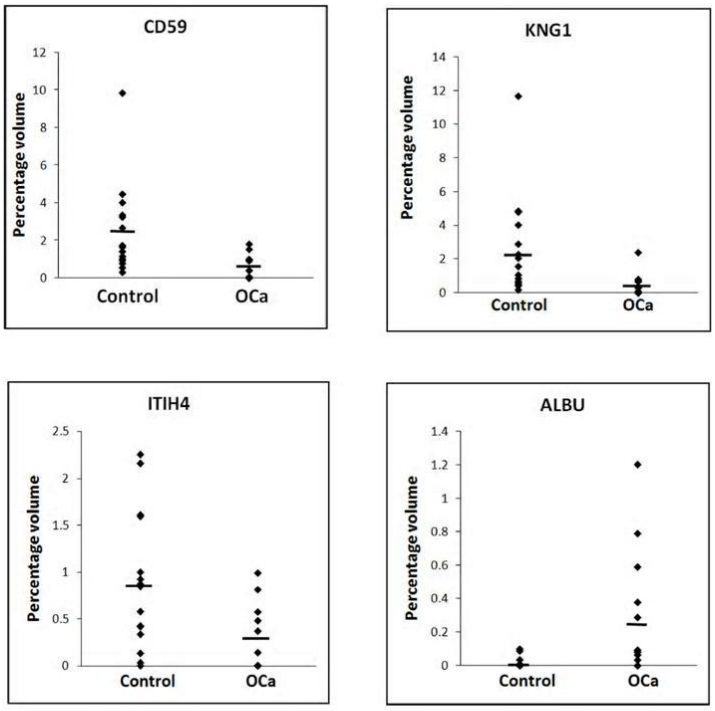

Figure 2 Relative excretion of urine proteins by control subjects and patients with ovarian cancer. The percentage of volume contribution was determined using the Image Master 2D Platinum Software 7.0. Image analysis performed on protein spot clusters that appeared consistently within each cohort of urine samples demonstrated the aberrant excretion of CD59, kininogen-1 (KNG1), ITIH4 (39 kDa fragment) and 19 kDa fragment of albumin (ALBUf) by patients with ovarian carcinoma (OCa).

[26], although the cause for the altered levels of kininogen-1 in these diseases may have been different.

The precise reason for the reduced levels of the ITIH4 fragment in the urine of patients with ovarian carcinoma that is observed in this study is currently not understood. The estimated molecular mass of the urine ITIH4 fragment indicated that it was slightly larger than its reported 35 kDa serum counterpart in the ovarian carcinoma patients [27]. Detection of the different sizes of ITIH4 fragments was not surprising as previous studies using SELDI-TOF-MS have demonstrated that ITIH4 was extensively processed within its proline-rich region in the human serum. In different diseases including ovarian carcinoma, different fragments were shown to be proteolytically generated [28]. While the present study demonstrated the reduced levels of the 39 kDa ITIH4 fragment in the urine of patients with ovarian carcinoma, our previous data showed the up-regulated levels of a 35 kDa ITIH4 fragment in the serum samples of the patients [27]. This inverse relationship and the difference in the molecular masses of the ITIH4 fragments detected in the respective samples suggest presence of a selective glomerular filtration mechanism that retained the 35 kDa fragment in the blood but allowed its 39 kDa counterpart to be excreted in the urine.

Table 3 Relative excretion of urine proteins in ovarian cancer

Urine proteins	Fold changes*	*p*
CD59	- 3.60	0.001
Kininogen-1	- 2.50	0.001
ITIH4[tf]	- 1.86	0.002
Albumin[tf]	+ 274.07	0.018

*Fold expression changes are relative to the control values

(-) decrease in expression; (+) increase in expression

[tf] Truncated fragment of protein

A *p* value of less than 0.05 is considered significant.

Based on their resolved locations in the 2-DE gels and MS/MS derived sequences, the enhanced albumin spots detected in the urine of ovarian cancer patients in this

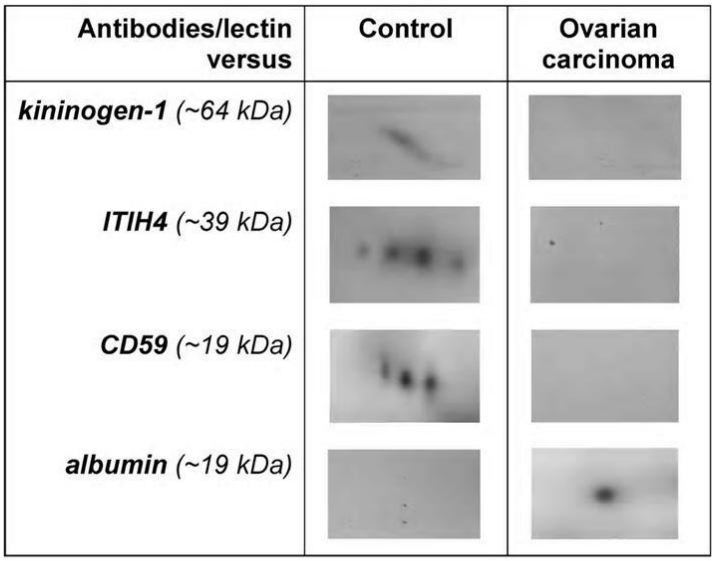

Figure 3 Interaction of antisera and CGB lectin with aberrantly excreted urine proteins. Pooled urine samples of ovarian cancer patients (OCa) and those of control subjects (Con) were subjected to SDS-PAGE and Western blotting before being independently exposed to antisera that bind to CD59, kininogen-1 and albumin as well as the ITIH4 binding CGB lectin.

study appeared to be fragments of albumin that consist of amino acids between positions 118 to 281, and with an approximate molecular mass of 19 kDa. The human urine is known to contain low levels of albumin fragments, with some polypeptides containing discontinuous sequences joined by unknown crosslinks [29]. Since the 19 kDa albumin fragment was present only in trace quantities in the urine of the control subjects, it may be used as a complementary urine biomarker to differentiate ovarian carcinoma patients from healthy individuals.

Conclusion

The proteomic profiling of urine samples demonstrated reduced levels of CD59, kininogen-1 and a 39 kDa ITIH4 fragment, as well as the enhanced excretion of a 19 kDa fragment of albumin in patients with ovarian carcinoma compared to control women. This observation may be applied in the development of noninvasive protocols for diagnosis and/or monitoring of the cancer.

Methods

Urine samples and processing

Urine samples were collected from patients newly confirmed with stages II and III ovarian carcinoma (n = 11), prior to treatment, at the University of Malaya Medical Centre (UMMC), Kuala Lumpur. All patients showed normal serum creatinine values. Control urine samples were collected randomly from age-matched cancer negative women (n = 15). Samples obtained were with consent and approval granted by the ethical committee of UMMC in accordance to the ICH GCP guideline and the Declaration of Helsinki. The subjects were of different ethnic background (Malay, Chinese and Indian). Sodium azide was immediately added to the urine upon collection to a final concentration of 20 mM. The samples were centrifuged at 10,000 rpm at 4°C and the supernatant was collected and dialyzed against distilled water. The urine proteins were aliquoted, freeze-dried and kept at -20°C. Protein content was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford USA).

Two-dimensional gel electrophoresis

IPG strips (pH 3-10, 11 cm) were rehydrated overnight in presence of 300 μg urine proteins in 200 μl rehydration solution (8 M urea, 0.5% v/v Pharmalyte 3-10, 0.5% v/v NP-40). Isoelectric focusing was performed using the Multiphor™ II Electrophoresis unit (GE Healthcare, Uppsala, Sweden) for a total of 12001 Vh at 20°C. The samples were then reduced by incubation of the strips in equilibrium buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% w/v DTT for 15 min prior to SDS-PAGE, and alkylated using 2.5% w/v iodoacetamide in the same equilibrium buffer

for another 15 min. The strips were then laid onto 12.5% polyacrylamide gels and electrophoresis was performed at 25 mA per gel.

Silver staining and image analysis

The 2-DE gels were developed by silver staining according to the method of Heukeshoven and Dernick [30] and scanned using the Image Scanner III. For mass spectrometric analysis, staining of gels was performed in absence of glutaraldehyde. Protein profiles were evaluated using the ImageMaster™ 2 D Platinum Software (Version 7). Image analysis was restricted to protein spot clusters that appeared consistently within each cohort of urine samples. The levels of proteins in each urine sample were evaluated as a percentage of volume contribution (%vol) to eliminate possible variations due to differential staining.

Mass spectrometry and database search

Protein spots of interest were excised from the silver stained gels and subjected to in-gel digestion according to the method of Shevchenko *et al.* [31]. Gel plugs were destained using 50 mM sodium thiosulphate: 15 mM potassium ferricyanide (1:1; v/v). Proteins in the plugs were reduced with 10 mM DTT in 100 mM ammonium bicarbonate for 30 min at 60°C, followed by alkylation with 55 mM iodoacetamide in the same solution for 20 min at RT in dark. The gel plugs were washed with 50% acetonitrile (ACN) in 100 mM ammonium bicarbonate, dehydrated by incubating in 50 μl ACN for 15 min and left to dry using a speed vac. Proteins were then digested with 7 ng/μl trypsin in 50 mM ammonium bicarbonate overnight at 37°C, extracted twice using 50% ACN and concentrated using the speed vac. The resulting peptide solutions were desalted and concentrated using zip-tips (Perfect Pure C18, Eppendorf, Hamburg, Germany). One μl aliquot was spotted onto a sample plate with 1 μl of matrix solution (α-cyano-4hydroxycinnamic acid, 10 mg/ml in 70% v/v ACN, 0.1% v/v TFA) and was allowed to air dry.

MALDI mass spectrometry was performed using the Applied Biosystems 4800 Proteomics Analyser. Spectra were initially acquired in reflecton mode in the mass range of 1000 to 4000 Da. The instrument was then switched to MS/MS (TOF/TOF). Ten strongest peptides from the MS scan were isolated, fragmented and reaccelerated to measure their masses and intensities. The data were exported in a format suitable for submission to the MASCOT database search program (Matrix Science Ltd., London, UK) and searched against 'all entries'. Identification was accepted when ≥ 5 peptide masses matched to a particular protein (mass error ± 50 ppm - 1 missed cleavage) and the MOWSE score was over the threshold score at $p = 0.05$.

SDS-polyacrylamide gel electrophoresis and Western blotting

Urine samples of patients with ovarian carcinoma (n = 11) and control subjects (n = 15) were separately pooled and subjected to unidimensional SDS-PAGE according to the method of Laemmli [32]. Gels consisting of 12.5% w/v acylamide were used. Separated proteins were transferred to nitrocellulose membranes (0.45 μm) using the NovaBlot Kit of Multiphor II Electrophoresis System (GE Healthcare, Uppsala, Sweden) at 0.8 mA/cm^2.

The membranes were blocked with 3% w/v gelatine in Tris-buffered saline (TBS), pH 7.5, for 1 h at RT and washed three times with the same buffer. They were then incubated for another 1 h in the following HRP-conjugate solutions: (1) anti-human CD59 (Abcam, Cambridge, UK - Cat. No. ab9182, at 1:5 dilution), (2) anti-human kininogen-1 (Abnova, Jhongli, Taiwan - Cat. No. H00003827-B01, at 1:500 dilution), (3) anti-albumin (Sigma Chemical Company, St. Louis, MO USA - Cat. No. A0433, at 1:40 dilution) and (4) champedak galactose binding (CGB) lectin (0.01 μg/ml) diluted/dissolved in TBST. The use of the CGB lectin to detect the C-terminal O-glycosylated ITIH4 fragment has been previously reported [27]. Development of the Western blot was performed using 25 ng 3,3'-diaminobenzidine and 5 μl 30% v/v H_2O_2 in 50 ml TBS. The reaction was stopped by washing the membranes with distilled water.

Statistical analysis

All values are presented as mean ± SD. The Student's *t*-test was used to analyze significance of differences between control subjects and patients. A p value of less than 0.05 was considered significant.

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Authors' contributions

SSAS carried out the experiments, analyzed the data and drafted the manuscript; BKL provided the urine samples; OHH contributed to the design of the study and critically revised the manuscript; ASS planned the study and critically revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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