PROTEOMICS-BASED IDENTIFICATION OF POTENTIAL PROTEIN BIOMARKERS FOR EPITHELIAL OVARIAN CANCER

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SUMMARY

Epithelial ovarian cancer (EOC) is one of the most common gynaecological cancers and is a leading cause of death in women worldwide. The current detection and prognostication protocols generally involve measuring serum CA-125 levels which have met with limited success. The identification of proteins released into the cyst fluid of EOC could provide the basis for the discovery of possible candidate protein markers with diagnostic and prognostic potentials.

Using the proteomics-based methods including surface enhanced laser desorption/ionisation time of flight (SELDI-TOF), two dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI TOF/MS), the differentially expressed haptoglobin and ceruloplasmin were identified in cyst fluid from malignant when compared with benign ovarian tumours. Validation of these biomarkers using traditional immunologic methods including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western analysis, immunocapture proteinChip analysis, immunohistochemistry and enzyme linked immunosorbent assay (ELISA) proved the validity of the two proteins as potential biomarkers. Diagnostic and prognostic significance of haptoglobin in serum as well as in cyst fluid from patients presenting with various stages of EOCs were evaluated. Although the serum haptoglobin had limited roles in pre-operative diagnosis of this disease, the study did provide evidence that pre-operative serum haptoglobin could serve as an independent prognostic factor in patients presenting with EOC. Our data indicated that elevated serum haptoglobin levels were associated with poor outcome for overall survival using both uni- and multivariate analyses.

The potential application in clinics using cyst fluid haptoglobin levels as an intra-operative diagnostic method was also tested. It showed that haptoglobin had an enhanced predictive performance when combined with CA-125 and ultrasound parameters as a preliminary study using 47 benign and 43 malignant ovarian tumours giving 88.4% sensitivity and 91.5% specificity with a PPV of 90.5% and NPV of 89.6% for EOCs. Such intra-operative cyst fluid determination of haptoglobin levels using a simple test kit with a specific cut-off value has potential clinical significance in that it could be performed as an adjunct to frozen section and be utilised to triage women requiring frozen section or sub-specialist consult, so that these services are more cost-efficient.

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LIST OF ABBREVIATIONS

µg	Microgram
µm	Micrometer
2-D DIGE	Two dimensional differential in gel electrophoresis
2-DE	Two dimensional gel electrophoresis
ACTH	Adrenocorticotropic hormone
AFP	Alpha-fetoprotein
APC	Adenomatous polyposis coli
ATCC	American Type Culture Collection
ATIII	Antithrombin III
AUC	Area under the curve
bp	Base pair
BPB	Bromophenol blue
BRCA1	Breast cancer 1 Early Onset
BRCA2	Breast cancer 2 Early Onset
BSA	Bovine serum albumin
CA125	Cancer antigen 125
CA72-4	Cancer antigen 72-4
CEA	Carcinoembryonic antigen
CI	Confidence interval
CID	Collision induced dissociation
cm	Centimetre
CRP	C-reactant protein
cSHMT	Cytosolic serine hydroxymethyl transferase
CV	Coefficients of variance
DCC	Deleted in colorectal cancer
DMSO	Dimethyl sulfoxide

DNA	Deoxy ribonucleic acid
DSRB	Domain Specific Review Board
DTT	Dithiothreitol
e.g.	Example
EAM	Energy absorbing molecule
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EOC	Epithelial ovarian cancer
ESI	Electrospray ionisation
FSH	Follicle-stimulating hormone
g	Centrifugal g force or grams
GTP	Guanosine triphosphate
H & E	Haematoxylin and eosin
HAP1	Haptoglobin-1 precursor
Hb	Haemoglobin
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IAA	Iodo-acetamide
ICAT	Isotope-coded Affinity Tags
IEF	Isoelectric focusing
IgG	Immunoglobin G
IL	Interleukin
IMAC	Immobilised affinity capture
kDa	Kilo Dalton
LCM	Laser capture microdissection

LH	luteinising hormone
LMP	Low malignancy potential
LOH	Loss of heterozygosity
LPA	Lysophosphatidic acid
m/z ratio	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ionisation
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage/monocyte colony stimulating factor
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
MOPS	Morpholinopropanesulphonic acid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MUC-1	Mucin 1
MudPIT	Multidimensional protein identification technology
MW	Molecular weight
Myc gene	Myelocytomatosis virus gene
NaCl	Sodium chloride
ND	Not detected
ng	Nanogram
NP20	Normal phase 20
NPV	Negative predictive value
ns	Nanosecond
OC 125	Ovarian cancer 125

OPN	Osteopontin
OSE	Ovarian surface epithelial cells
PBS	Phosphate buffered saline
PCR-SSCP	Polymerase chain reaction single strand conformation polymorphism
pg	Picogram
pI	Isoelectric point
PID	Pelvic inflammatory disease
ppm	Parts per million
PPV	Positive predictive value
PTM	Posttranslational modification
PVC	Polyvinylchloride
RAS	Rats sarcoma gene
RhoGDI	Rho G-protein dissociation inhibitor
RMI	The risk of malignancy index
ROC	Receiver operating characteristics
RP	Reversed phase
rpm	Revolutions per minute
RPMI	Roswell Park Medical Institute
rRNA	Ribosome ribonucleic acid
RT	Reverse transcription
SAA1	Serum amyloid A1
SAX	Strong anion-exchange
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELDI	Surface enhanced laser desorption/ionization
sec	Second
TBS	Tris buffered saline
Tbx3	T-box transcription factor 3

TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β	Transforming growth factor-β
TNF-a	Tumour necrosis factor-a
TOF	Time-of-flight
TP53	Tumour protein 53
tPA	Tissue-type plasminogen activator
TTR	Transthyretin
U	Unit
uPA	Urokinase-like plasminogen activator
UV	Ultraviolet
V	Volt
VEGF	Vascular epidermal growth factor
Vh	Volt hour
WCX	Weak cation exchange

Chapter 1: Introduction

1.1 Overview

1 in 75 women will develop ovarian carcinoma sometime during their lifetime (Holschneider et al., 2000). Worldwide there are 204 449 new cases of ovarian cancer diagnosed annually, and an estimated 124 860 disease-related deaths (IARC, 2006). Epithelial ovarian cancer is now the leading cause of gynaecological cancer-related deaths in the UK and the USA (Bristow et al., 2006). In Singapore, ovarian cancer is the most common gynaecological malignancy and the fourth most common female cancer (Figure 1.1). The incidence of this cancer saw a sharp rise from 222 cases in 1968-1972 to 1055 cases in 1998-2002 (Seow. et al., 2004). The age-adjusted rate of incidence for ovarian cancer was 6.0/100,000 in 1968-1972 and rose to 11.0/100,000 (1998-2000, Table 1.1).

Despite the progress in cancer therapy, ovarian cancer mortality has remained virtually unchanged over the past two decades. This is attributed to the difficulties in early diagnosis and therefore, ovarian cancer has the highest mortality rate of all the gynaecological cancers (Kristensen et al., 1997). The overall survival rate of ovarian cancer is about 50% over a 5-year period, and this is largely dependent upon the stage of the disease at the time of diagnosis. However, early diagnosis of this cancer results in a 5-year survival rate of about 80% (Kristensen et al., 1997). Regular pelvic examinations and CA-125 measurements followed by radiological diagnosis on an individualised basis have been the current practice for detection of this enigmatic condition. However, neither an elevated serum CA-125 level, nor the presence of an ovarian cyst

identified by clinical examination and ultrasonography, accurately predicts the occurrence of an ovarian malignancy (van Nagell et al., 2000).

Recent development in genomic and proteomic technology has made it possible to apply high throughput methods to detect alterations in gene and protein expression and their association with disease processes (Welsh et al., 2001; Zhang et al., 2004). In this context, many polypeptides have been identified to be highly expressed in tumours with potential clinical utilities. However, there is still a dearth of clinically useful markers for the diagnosis as well as prognostication of ovarian cancer.

In this thesis, I explore the possibility of identifying differentially expressed protein biomarkers in ovarian cyst fluid using combined proteomics-based methods. This fluid represents a source of potential significance in the identification of target markers since the protein composition changes occurring in EOC cells will be probably reflected in the cyst fluid. The exploration of secretion and expression of these polypeptides may revolutionise the way of diagnosis and prognostication of ovarian cancer patients.

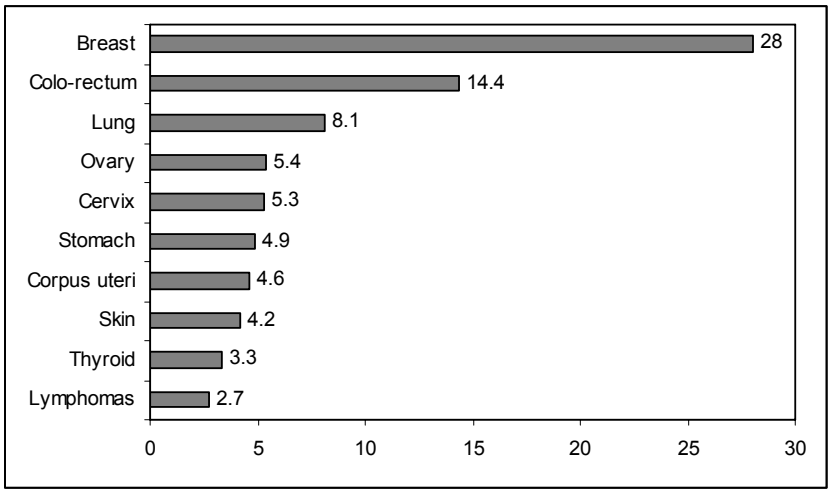


Figure 1.1 Ten most frequent cancers in females in Singapore, 1998-2002.
Adapted from Seow, (2004)

Table 1.1 Incidence of ovarian cancer in Singapore, 1968-2002

Adapted from Seow, (2004)

Year	No.	Age-standardised rate (per 100,000/year)
1968-1972	222	6.0
1973-1977	263	6.3
1978-1982	411	8.6
1983-1987	497	8.8
1988-1992	702	10.5
1993-1997	880	11.4
1998-2002	1055	11.0

1.2 Ovarian cancer

1.2.1 Aetiology

The exact aetiopathogenesis of ovarian cancer remains poorly understood. Current research studies have focused on the reproductive, genetic and environmental influences in the carcinogenesis of this insidious disease.

1.2.1.1 Reproductive and endocrine factors

Increasing epidemiological studies have indicated that reproductive/hormonal milieu could be responsible for the high risk of ovarian cancer in postmenopausal women. The levels of follicle-stimulating hormone (FSH) and luteinising hormone (LH) reach the highest level in menopausal period. These gonadotropins together with other steroid hormones such as oestrogen, progesterone and androgen are believed to be involved in the ovarian tumourigenesis (Risch, 1998; Ho, 2003). Oestrogen has long been regarded as a causative factor with its level in ovarian tissue being at least 100 times higher than that in blood. It is envisaged that the high hormone levels could enable a direct genotoxic effect on ovarian surface epithelial (OSE) cells (Ho, 2003). In addition to the genetic damage, studies showed that oestrogen might increase the risk of ovarian cancer by oestrogen receptor–mediated growth stimulatory responses due to the fact that expression of oestrogen receptor has been observed in both OSE and ovarian tumour cells (Karlan et al., 1995; Lau et al., 1999; Syed et al., 2001). Binding of oestrogen to the receptor could result in cellular proliferation which has been demonstrated in several oestrogen positive ovarian cancer cell lines (Langdon et al., 1994). Administration of a competitive agent such as tamoxifen could significantly inhibit this growth stimulatory effect (Nash et al., 1989).

Moreover, numerous oestrogen-regulated proteins have been studied in the multiple carcinogenesis process of ovarian cancer. These proteins such as cyclin D1, kallikreins and cathepsin-D are involved in cellular growth, motility and invasion of the malignant cells (Clinton et al., 1997; Rochefort et al., 2001).

The so called “incessant ovulation” mechanism is considered to be another main aetiological factor for ovarian carcinogenesis, which was firstly introduced in 1971 due to relatively high risk of ovarian cancer in nulliparous women (Fathalla, 1971). Most of ovarian cancers originate from OSE cells which cover the entire surface of ovary and present as a single layer of cuboidal, low columnar or flattened cells (Blaustein et al., 1979). It was hypothesised that after repeated ovulation, the repairs of trauma in surface epithelium somehow resulted in aberrant proliferation and malignant transformation of the OSE cells. This theory was also supported by the increased incidence of ovarian cancer in the patients subjected to hyperovulation by drugs (Fathalla, 1971) and protective effects of parity and oral contraceptive (Rodriguez et al., 1998). Moreover, a study showed that there was a marked increase of the apoptotic epithelial cells after treatment with contraceptive in animal models (Rodriguez et al., 1998). This finding suggested that oral contraceptive might prevent development of ovarian cancer by rendering the cells which are prone to be malignant to undergo apoptosis.

1.2.1.2 Hereditary factors

Although familial neoplasm only accounts for a small proportion of all ovarian cancers when compared with the sporadic form of the disease, a strong family history for malignancy indicates a genetic predisposition to ovarian cancer. Women with a first-degree relative with ovarian cancer may have as high as 50% chance of developing this familial disease compared to only 1.4% chance for

those without a family history (Schildkraut et al., 1988). For patients with breast cancer, the relative risk of developing ovarian cancer varies from 0.6 to 6.1 (Hildreth et al., 1981; Cramer et al., 1983a; Koch et al., 1988). Inheritance of mutated tumour suppressive genes BRCA1 and BRCA2 is believed to have an important role in development of familial ovarian cancer. Mutations such as point mutation, gene amplification and chromosomal translocation in these genes result in loss of function of tumour-suppressive abilities, which is considered as the first somatic genetic event driving the development of ovarian cancer (Auersperg et al., 1998). The proteins encoded from these genes function in transcriptional activation and DNA repair, some of which are key regulators in maintaining the balance of cellular proliferation and apoptosis (Auersperg et al., 1998).

For sporadic ovarian cancer, which accounts for more than 95% of the cases, few mutations of BRCA1 and BRCA2 have been detected, indicating the presence of genetic changes in other oncogenes and tumour-suppressor genes (Gallion et al., 1995). Hence, substantial efforts have been directed to determine the altered region in genome and abnormal genes with lost functions based on mutation or differential expression study in tumour specimens. More than 60 deregulated genes have been found in ovarian cancer including HER-2/neu, K-ras, c-myc with varying frequency (Aunoble et al., 2000), most of which encode proteins that are involved in growth stimulatory pathways in malignant as well as normal cells. Activation of these genes due to amplification or mutations has been regarded as a causative factor in the carcinogenesis of ovarian cancer. For example, It was reported 32% of ovarian cancer overexpressed HER-2/neu, a gene which encodes cell membrane receptors involved in transmitting growth stimulatory signals when compared with normal ovary (Berchuck et al., 1990).

1.2.1.3 Environmental factors

The relatively high incidence of ovarian cancer in industrialised countries suggests that environmental factors or diet may be involved in its aetiology. Excessive dietary intake of animal fat or red meat have been reported to increase the risk of EOC (Byers et al., 1983; Shu et al., 1989), while dietary fish and vegetables have been suggested to have a protective role (La Vecchia et al., 1987; Shu et al., 1989). The case-control study conducted in 1990 indicated a positive correlation of increasing consumption of milk with high risk of ovarian cancer (Mettlin et al., 1990). Moreover, the reports on the association of mumps infection and risk of ovarian cancer have been conflicting. Cramer and colleagues found an close relation between childhood mumps and subsequent ovarian cancer (Cramer et al., 1983b). However, another group from Israel observed low titres mumps antibody in ovarian cancer patients indicating a weak aetiological association of this virus with this neoplasm (Menczer et al., 1979).

1.2.2 Pathology classification

Epithelial ovarian tumours account for approximately 60% of all ovarian tumours and their malignant forms account for more than 90% of all ovarian cancers (Russell, 1979). According to histologic differentiation, the EOC is classified into four major groups: serous, mucinous, endometrioid and clear cell tumours. The malignant tumours usually have the similar histologic architecture of the endocervix for mucinous cancer, the endometrium for endometrioid cancer and the fallopian tubes for serous cancer (Russell, 1979). They are also classified as benign tumours, tumours of low malignant potential (LMP) and malignant tumours based on cytological features and clinical behaviour. Tumours of low malignant potential are also named as borderline tumours. This group of tumours is

characterised by the morphologic features intermediate between benign and malignant forms of ovarian tumours. More than 70% of borderline tumours show localised disease at presentation which favours a better prognosis compared to malignant diseases.

Serous cancers are the most common histologic type of ovarian malignancies, accounting for approximately 50% of all EOCs (Russell, 1979). These tumours are usually bilateral and present as large, uni or multilocular cyst with the diameter greater than 5 cm in majority of cases. Most tumours are partially cystic and partially solid. The solid area with haemorrhage or necrosis is the general feature indicating malignant potential of the tumours. Microscopically well-differentiated serous cancer is characterised by presence of papillae covered with stratified epithelial cells. However, it is only focally present in poorly differentiated serous malignancies. Borderline and malignant mucinous tumours represent the second most common type, accounting for 10% to 20% of all EOCs (Russell, 1979). They are usually large, cystic and multiloculated tumours with viscous mucin filled inside, the outer surface of which are generally smooth with occasional papillae projection, although some EOCs present as solid masses or predominantly solid tumours which disseminate widely throughout the peritoneal cavity. Endometrioid cancer has the same histologic appearance as a typical endometrial cancer, accounting for 10% to 25% of all EOCs (Czernobilsky et al., 1970). Most endometrioid cancers are cystic, frequently with papillary structures filling the lumen. Clear-cell cancer is the most chemoresistant type and accounts for about 5% of epithelial ovarian carcinoma (Kurman et al., 1972). Most tumours are partially cystic. Microscopically it is characterised by hobnail-shaped tumour cells with cleared-out cytoplasm (Kurman et al., 1972).

There are two important prognostic factors for ovarian cancer—stage and grade. The tumour stage describes how far the tumour has spread from where it starts in the ovary. Staging is explained in detail by FIGO in 1985 (Gynecol Oncol, 1986; Table 1.2). Clinical studies indicate that staging of ovarian cancer has significant impact on the survival of this disease. The five-year survival rate shows a dramatic increase from 15 to 20% in late stage cancers to 90% for stage I patients (Young et al., 1990). Histologic grade is also an important prognostic factor for ovarian cancer. The grade is on a scale of 1, 2, or 3 representing well, moderate and poor differentiation respectively. Grade 1 epithelial ovarian carcinomas more closely resemble normal ovarian tissue in contrast with those from grade 3. Studies indicated that well-differentiated (G1) ovarian cancer tends to have a better prognosis compared to poorly-differentiated (G3) ones (Einhorn et al., 1985; Swenerton et al., 1985).

Table 1.2 FIGO staging for primary carcinoma of the ovary

Stage		Description
I		Growth limited to the ovaries.
	IA	Growth limited to one ovary; no ascites. No tumour on the external surface; capsule intact.
	IB	Growth limited to both ovaries; no ascites. No tumour on the external surface; capsule intact
	IC	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.
II		Growth involving one or both ovaries with pelvic extension.
	IIA	Extension and/or metastases to the uterus and/or tubes.
	IIB	Extension to other pelvic tissues
	IIC	Tumour either Stage IIA or IIB, 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
III		Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals Stage III. Tumour is limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum.
	IIIA	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces
	IIIB	Tumour of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces not exceeding 2 cm in diameter. Nodes are negative.
	IIIC	Abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
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.

1.2.3 Pre-operative Diagnosis of EOCs

A key challenge in cancer control and prevention is the detection of the disease at an early stage, which could result in effective intervention and contribute to reduction in mortality and morbidity. Unfortunately, the early diagnosis of ovarian cancer is difficult because of the physically inaccessible location of the ovaries, the lack of specific symptoms in the early stage of disease, and the limited understanding of ovarian carcinogenesis (Cannistra, 2004). Because currently available screening tests do not achieve high enough levels of sensitivity and

specificity to be useful for the detection of this low prevalent disease, screening is not recommended for the general population (Jacobs et al., 2004).

1.2.3.1 Signs and symptoms

The early diagnosis of ovarian cancers is difficult because symptoms arising from neoplasm are often absent or vague until the tumours become large and have metastasized. Retrospective studies indicated that women with ovarian cancer presented with non-specific symptoms including pelvic or abdominal discomfort, urinary frequency, and alterations in gastrointestinal functions (Flam et al., 1988; Goff et al., 2000; Olson et al., 2001). There are no specific symptoms indicating early stage of ovarian cancer and patients with non-specific symptoms are always misdiagnosed as diseases from other systems. Ovarian tumours are usually palpable in pelvic examinations of postmenopausal women, while palpable tumours in premenarchal girls could indicate the presence of malignancy (Shankar et al., 2001).

1.2.3.2 Blood testing

Measurement of serum cancer antigen 125 (CA-125) levels in blood has been widely employed in the detection of ovarian cancer. Concentrations of CA-125 were elevated in approximately 80% of patients presenting with advanced ovarian cancer whereas only in 50% of patients presenting with stage I disease (Bast et al., 1985). Moreover, elevated concentration of CA-125 was associated with various kinds of malignancies such as pancreas, breast and ovary cancers (Bast et al., 1983). It was also observed to be elevated in benign conditions such as endometriosis and pelvic inflammatory disease (PID) (Daoud et al., 1991).

Despite the poor sensitivity and specificity, CA-125 is mainly useful in monitoring therapeutic effects and recurrence of this insidious disease (Jacobs et al., 1989).

1.2.3.3 Ultrasonography evaluation

Ultrasonography may be used to differentiate malignant from benign ovarian tumours. Malignant ovarian tumours are generally multiloculated and have more solid areas with thick septa. Ascites and metastasis present in malignancies are also easily identified by ultrasound assessment. Several attempts have been made to distinguish early stage ovarian cancers from questionable ovarian cyst accurately based on greyscale ultrasound and colour Doppler features (Kurjak et al., 1993; Fleischer et al., 1996).

The risk of malignancy index (RMI) scoring systems was developed from a combination of ultrasound and CA-125 concentrations (Table 1.3). It is the method of choice for predicting whether or not an ovarian mass is likely to be malignant. There are three scoring systems, RMI1, RMI2 and RMI3 which are shown in Table 1.3 (Jacobs et al., 1990; Tingulstad et al., 1996; Tingulstad et al., 1999). The modified RMI 2 and RMI 3 scoring methods are more sensitive compared with RMI 1 method, resulting in a better sensitivity and specificity in diagnosis of ovarian malignancies (Tingulstad et al., 1996; Tingulstad et al., 1999; Aslam et al., 2000).

Table 1.3 The RMI scoring system

Feature	RMI 1 Score	RMI 2 Score	RMI 3 Score
Ultrasound features • multilocular cyst • solid areas • bilateral lesions • ascites • metastases	0 = none 1 = one abnormality 3 = two or more abnormalities	1 = none or one 4 = two or more abnormalities	1 = none or one 3 = two or more abnormalities
Premenopausal	1	1	1
Postmenopausal	3	4	3
CA-125	U/ml	U/ml	U/ml

1.2.4 Management of ovarian cancer patients

In ovarian cancer, surgery usually involves total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and pelvic and para-aortic lymphadenectomy (Cannistra, 2004). In borderline tumours, a fertility sparing approach can be used, particularly in patients who desire fertility preservation (Robinson et al., 1992). Accurate differential diagnosis between malignant and benign ovarian tumours is therefore crucial, and has important implications for both the intra-operative and post-operative management of every case. Appropriate staging and surgery for all stages of EOC has been shown to have a direct impact on patient survival. A population-based study done by Engelen et al (2006) showed that the risk of dying for patients who did not undergo appropriate surgery was twice the risk of those who underwent adequate and appropriate surgery. However, definite histopathologic diagnosis is usually revealed at least a few days after surgical removal, using the standard technique of paraffin section. As a result, inadequate surgical removal of the tumour and surrounding tissue is a potential problem in ovarian cancer patients.

1.2.5 Intra-operative diagnosis of EOCs

Intra-operative diagnosis for the presence or absence of malignancy in an ovarian cyst is important in determining the choice and extent of surgical procedures. Intra-operative suspicion of malignancy is particularly important in younger patients wanting to preserve fertility (Palomba et al., 2007), and in patients undergoing laparoscopic surgery for ovarian pathology (Chapron et al., 1998). An ovarian malignancy is unexpectedly encountered in up to one percent of cases undergoing laparoscopy (Wenzl et al., 1996; Muzii et al., 2005). Neither an elevated serum CA-125 level, nor the presence of an ovarian cyst identified by clinical examination and ultrasonography, accurately predicts the occurrence of an ovarian malignancy (van Nagell et al., 2000). Benign and malignant tumours cannot always be accurately and easily distinguished visually (Lim et al., 1997) and even the absence of solid areas or septae within ovarian cysts, and negative findings from intra-operative video-laparoscopic surveillance cannot entirely exclude the possibility of ovarian malignancy (Wenzl et al., 1996).

Currently, frozen section examination of tumour tissue is the mainstay of intra-operative diagnosis for the benignity or malignancy of an ovarian cyst. The accuracy of frozen-section results is a determinant in the selection of the appropriate surgical procedure to prevent under- and over-treatment for ovarian cancer patients and provides important information on patient prognosis (Yeo et al., 1998). Unfortunately, frozen section services are not universally and uniformly available. It requires a trained pathologist to be on site and on standby. Besides being expensive and resource intensive, many centres do not offer this service after office hours, while in many emerging economy countries most centres do not offer it at all. Frozen section analysis is also limited by the selection of sampling location. It is affected by the large size of some tumours,

the heterogeneous composition, and an often focal nature of the malignancy (Rose et al., 1994; Yeo et al., 1998; Pinto et al., 2001). In comparison with other gynaecologic conditions, frozen section diagnosis of ovarian tumours has also been reported to be most problematic in terms of its accuracy, achieving a sensitivity of 85.7 %, specificity of 87.2 %, positive predictive value (PPV) of 89.4 % and negative predictive value (NPV) of 82.9 % (Boriboonhirunsarn et al., 2004). Such levels of diagnostic accuracy fall short of the ideal in cancer diagnostics. An alternative method for intra-operative diagnosis, which is easier, cheaper, faster, and at least as accurate as frozen section would clearly be very useful.

Identification of a specific protein biomarker within biological materials that can be obtained during surgery such as ovarian cyst fluid would be useful. This may help to either diagnose or create a high level of suspicion for malignancy. It could allow the development of a rapid intra-operative identification test for women with possible ovarian malignancy.

1.3 Biomarkers for ovarian cancer

At present, there is a dearth of clinically useful markers for the diagnosis as well as prognostication of ovarian cancer. The currently used serum marker CA-125 has limited sensitivity and specificity for detecting small volume, early stage disease, with a PPV of less than 10% (Jacobs et al., 2004).

The malignant transformation of a normal ovarian epithelial cell is caused by genetic alterations that disrupt the regulation of proliferation and apoptosis, in turn leading to altered protein expression and modification (Boyd et al., 1990; Weinberg, 1996). Genetic alterations and changes in protein levels could prove to be vital for detection of subjects at risk of developing cancers as a normal cell progresses through the complex process of transformation to a cancerous state. The changes which occur exclusively in cancer but not in the surrounding non-malignant cells, can be detected within the cancer cells and in the peripheral blood or body fluids (Srinivas et al., 2001) (Figure 1.2). Such genetic and proteomic biomarkers can serve as molecular signposts of the pathophysiologic state of a cancer cell, as well as for the diagnosis, prognostication and assessment of therapeutic response of the cancers.

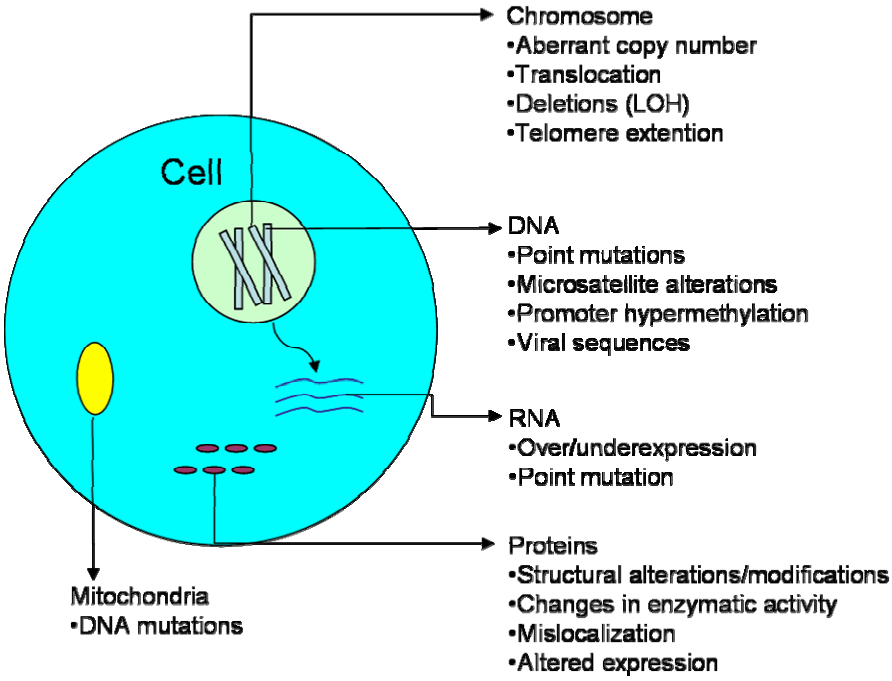


Figure 1.2 Molecular marker detection from cancer cells

Cancer cells may enter into blood and other body fluid as an epithelial tumour grows. They may also secrete proteins directly into body fluids. This makes it possible to detect genetic and protein biomarkers with diagnostic and prognostic potential for this cancers. There are different types of biomarkers which could be detected. DNA can be analysed for changes in gene copy number, mutation, loss of heterozygosity (LOH), microsatellite instability, and promoter hypermethylation. RNA can be analysed for expression levels, and proteins can be analysed for changes of expression level or localisation.

1.3.1 The genetic markers

All cancers are genetic in origin, in the sense that the driving force of malignant transformation is genetic variation (Boyd et al., 1990; Weinberg, 1996). The sequential mutations of cancer-related genes, with the subsequent selection and accumulation in a clonal population of cells, are the main factors determining whether a tumour develops and the time required for its development and progression (Boyd et al., 1990; Vogelstein et al., 1993). The most compelling evidence is that for human epithelial tumours, the incidence rate increases at fourth to eighth power in an age-specific manner, suggesting the presence of four to eight rate-limiting genetic alteration process in the development of cancers (Renan, 1993).

Genetic modifications, which are generally identified in early stages of cancers could lead to investigations on their role in the detection of human neoplasms. Identification of free circulating DNA provides the good source for searching genetic biomarkers for molecular diagnosis. Free DNA was initially identified 50 years ago in the serum or plasma from patients with cancers, and elevated levels of DNA were found in the serum of patients with metastatic diseases (Leon et al., 1977; Shapiro et al., 1983). The mechanism by which the DNA is released and survives in the circulation is not well understood. Plasma or serum DNA from cancer patients is being used to detect suitable tumour markers such as oncogene mutations and hypermethylation of the promoter regions (Ramirez et al., 2003; Gautschi et al., 2007). However, the specificity of serum or plasma DNA is not satisfactory since genetic changes of circulating DNA have also been observed in patients with other diseases (Lo et al., 2000; Chang et al., 2003). DNA can also be easily isolated from other body fluids such as saliva, urine and

stools, which could be useful in detecting specific tumour types (Lichtenstein et al., 2006; Quinque et al., 2006; Zou et al., 2006).

1.3.1.1 Gene mutations

Cancer-associated mutations were initially studied in solid tumours in the early 1990s, when TP53 mutations were identified in the urine from patients presenting with bladder cancer (Sidransky et al., 1991) and ras mutations in the stools from patients with colorectal cancer (Sidransky et al., 1992). This led researchers to search for other gene mutations that could be of use in cancer detection and prognostication.

The breast cancer susceptibility genes BRCA1 and BRCA2 are the most important ones known to predispose ovarian cancer. Various functions have been attributed to the BRCA tumour suppressor genes including regulation of cell cycle progression, induction of apoptosis, involvement in DNA repair and the maintenance of genomic integrity (Powell et al., 2003). Mutations in BRCA genes cause a high lifetime risk of both breast and ovarian cancer; the risk of ovarian cancer in BRCA1 mutation carriers is 40% by age 70, with the corresponding risk in BRCA2 carriers being about 10%. Mutations in these genes account for 5–13% of ovarian cancer cases in Western countries (Takahashi et al., 1995; Stratton et al., 1997; Rubin et al., 1998; Risch et al., 2001). Somatic mutations in BRCA1 are rarely identified in the sporadic tumours (Geisler et al., 2002). On the other hand, decreased expression of BRCA1 in sporadic ovarian and breast tumours has been observed and may contribute to the pathophysiology of this disease (Russell et al., 2000; Zheng et al., 2000).

While there exists clear evidence of mutation associated tumour characteristics such as histology, grade and stage for breast cancer, reports on sporadic ovarian carcinomas are conflicting. Some studies reported that papillary serous adenocarcinoma was the prominent BRCA1 mutation associated ovarian cancer type, while others report no such association (Chang et al., 1995; Rubin et al., 1996; Johannsson et al., 1997; Pharoah et al., 1999; Chen et al., 2003; Lakhani et al., 2004). There has been further suggestion that the BRCA1 status may influence patient survival (Rubin et al., 1996; Boyd et al., 2000; Cass et al., 2003).

Another genetic lesion that has been implicated in the genesis and development of ovarian cancer involves p53 tumour suppressor gene. The p53 gene located on chromosome 1; its various types of mutations have been observed in many human cancers (Soussi et al., 1994). In ovarian cancer, it has been observed to be mutated in about 30—50% of the patients (Marks et al., 1991; Okamoto et al., 1991; Tsao et al., 1991). The translational product of the p53 gene is a nuclear phosphoprotein involved in the regulation of normal cellular growth and development. The loss of normal p53 function, due to mutation, overexpression or deletion of the gene, is often associated with a malignant phenotype (Basset-Seguin et al., 1993; Soussi et al., 1994). It has been reported that P53 gene mutation is useful not only in detecting cancer, but also in monitoring therapeutic effects and determining disease prognosis (Laframboise et al., 2000; Havrilesky et al., 2003).

1.3.1.2 Loss of heterozygosity (LOH)

All cells contain two copies of autosomal genes inherited from each parent. According to Knudson's 'two kit' theory, both alleles of a growth-regulatory 'tumour suppressor' gene have to be altered either by mutation or deletion in

order to inactivate it (Knudson, 1971). If a cell develops a mutation in one allele of a tumour-suppressor gene, loss of the remaining wild-type allele — LOH — can initiate tumourigenesis (Obata et al., 2000). LOH can be detected by various PCR based approaches in most neoplasm including ovarian cancer. For example, chromosomal 17 was subjected to LOH in 50% to 80% of all ovarian cancer (Presneau et al., 2005). LOH was related with many tumour suppressive genes. High frequency deletion on the short arm of chromosome 17 was often associated with TP53 mutation (Phillips et al., 1996). Moreover, studies indicated that on 17q 78% of the deletion occurred at 17q 21 intragenic to BRCA1, 58% at 17q11.2 including NF1, and 46% at a locus distal to BRCA1, suggesting that multiple tumour suppressor genes involved in ovarian cancer on 17q (Tangir et al., 1996). The other well-established LOH studies on ovarian cancer included chromosome 5q and 18. The analysis of 49 ovarian tumours for LOH on chromosome 5q showed 47% of them had loss at 5q13.1-21 region proximal to APC. The losses were also observed in 33% of stage I tumours indicating involvement of genes within this region in the early carcinogenetic process of ovarian cancer (Tavassoli et al., 1996). On chromosome 18, LOH and mRNA expression of the DCC gene have been examined in ovarian tumours. It was envisaged that the significant loss or reduction of DCC expression in malignant tissues suggested a role for this tumour suppressor gene in tumour development (Enomoto et al., 1995). Hence, the characteristic LOH are valuable clonal markers for ovarian cancer.

1.3.1.3 DNA methylation

Methylation status in promoter regions of cancer-related genes has been reported to be another type of biomarker (Esteller et al., 2002). Loss of methylation in promoter region has two consequences in tumourigenesis. Firstly,

a weakening of transcriptional repression in normally silent regions of the genome could result in overexpression of the potentially harmful viral genes and normally silenced genes (Walsh et al., 1998). Secondly, losses of methylation would affect nuclear structures leading to chromosome instability in cancer. Specific PCR assay was developed to detect methylation status of cancers. This assay could identify one cancer cell out of 1,000 normal cells and is sensitive enough to detect tumour DNA in most of the body fluid (Herman et al., 1996).

On the other hand, hypermethylation of CpG islands in gene promoter regions is associated with aberrant silencing of transcription and regarded as a contributing factor in inactivation of tumour suppressor genes (Esteller et al., 2001). Groups of hypermethylated genes have been reported to be associated with ovarian cancer. For example, the hypermethylation profiles of several tumour suppressor and cancer-related genes (p16, RARbeta, E-cadherin, H-cadherin, APC et al.) in ovarian epithelial neoplasms were investigated with a view to better understand the role of epigenetic silencing in carcinogenesis (Makarla et al., 2005). It was found that all of these genes were hypermethylated in invasive ovarian cancers at a frequency of 9% to 30%. Three genes exhibited higher methylation frequencies in invasive tumours including RASSF1A (30%), H-cadherin (22%) and APC (22%) (Makarla et al., 2005). The frequent occurrence of aberrant methylation in invasive epithelial ovarian carcinomas therefore suggests that an accumulation of events at specific genes may trigger malignant transformation of benign and borderline tumours. Aberrant DNA methylation was also recognised as biomarkers in predicting disease free survival for ovarian cancer (Wei et al., 2002). Using specified microarray techniques, a panel of candidate methylated loci demonstrated the capability of predicting disease free survival with 95% accuracy (Wei et al., 2006).

1.3.1.4 Changes in gene expression

The most common mechanisms for mutational activation of human oncogene are gene amplification, typically resulting in overexpression of otherwise normally expressed protein products. On the other hand, the protein products of tumour suppressor genes functioning to inhibit cell proliferation are inactivated through loss-of-function mutations.

cDNA microarray has been used as a tool to study the mRNA expression profile in tumours as well as in normal tissues. A key emphasis in many of these studies has been the identification of gene products that could act as ovarian cancer specific biomarkers (Ismail et al., 2000; Wang et al., 2000; Welsh et al., 2001; Bonome et al., 2005). Using Affymetrix, U95 A Chip containing 12,000 genes, Warrenfeltz S and coworkers have come up with a genetic profile of ovarian cancer which could serve as a useful prognostic tool (Warrenfeltz et al., 2004). The profile, consisting of 115 genes, was found from 68 patient samples presenting with advanced ovarian cancer. The 115 genes were also biologically interesting, as many of the genes were previously shown to be involved in cancer growth, angiogenesis and many of them encode growth factors or their specific receptors. Another interesting study was conducted by Wong and colleagues. In their investigation, 30 differentially expressed genes in ovarian cancer were identified, among which osteopontin was detected as a putative biomarker (Wong et al., 2001). In a follow-up study, osteopontin was shown to be elevated at the protein level in plasma of ovarian cancer patients (Kim et al., 2002) indicating a positive correlation between transcription and translation process.

1.3.2 The protein markers

Cancer, although often classified as a genetic abnormality, is in a functional sense, a proteomic disease (Bishop, 1995). Genetic mutations can modify protein signalling pathways and thereby create a survival advantage for the cancer cells due to suppression of the inhibitory signals. While studies involving genomics and mRNA expression will continue to contribute substantially to medicine, there are limits to the type and amount of information such investigations can provide. Expression of mRNA and protein are not highly correlated by comparison of gene transcripts and the corresponding translational products (Celis et al., 2000; Steiner et al., 2000). In addition to providing quantitative data, the proteomics technology can provide additional qualitative information which includes post-translational modification and protein-protein interaction.

1.3.2.1 Protein markers in cyst fluid

Calgranulins

Calgranulins A and B, both low molecular weight proteins, were found to be overexpressed in the cyst fluid of malignant epithelial ovarian cysts (Ott et al., 2003). These two proteins are members of the S100 Calcium-binding protein family. They function as inhibitors of casein kinases I and II that are essential for phosphorylation of various molecules necessary for normal transcription and translation. The amount of detectable calgranulins was also found to correlate with tumour progression, i.e. early-stage ovarian carcinomas express much less calgranulins than advanced carcinomas, based on differences in the intensity of spots corresponding to the calgranulins on two-dimensional gel electrophoresis (Ott et al, 2003). However, no published studies to date have quantitatively

studied the intracystic expression of calgranulins or looked at its accuracy and usefulness in diagnosing ovarian malignancy in the clinical setting.

Growth factors and cytokines

Vascular endothelial growth factor (VEGF) has also been reported to be significantly over-expressed ($p<0.001$) in cyst fluid of malignant epithelial ovarian cysts compared to benign ovarian cysts (Demirkiran et al., 2003). VEGF is an important modulator of angiogenesis; it increases microvascular permeability and endothelial cell growth. However, no statistically significant correlation between cyst fluid VEGF levels and cancer grade and stage has been reported (Boss et al., 2001). Insulin-like growth factor-1 and IGF-binding protein-2, which are expected to have a mitogenic effect on the epithelial ovarian cancer cells, have also been found to be over-expressed in the cyst fluid of malignant ovarian cysts (Karasik et al., 1994).

A number of cytokines have also been studied for their expression in cyst fluid. Tumour necrosis factor (TNF), interleukin-6 (IL-6) and interleukin-8 (IL-8) have all been shown to be elevated in the cyst fluid of malignant epithelial ovarian cysts (Darai et al., 2003). The levels of IL-8 in malignant cyst fluid were, in a study, shown to be 13-fold higher than in benign cyst fluid (Ivarsson et al., 1998). IL-8 is known to influence proliferation and angiogenesis of several non-ovarian tumours.

Soluble cell-cell adhesion molecules

Adhesion molecules are important in carcinogenesis, as their abnormal expression by neoplastic cells may be an important determinant of local invasion and metastatic dissemination (Darai et al., 1998), A study by Sundfeldt et al showed, using an ELISA method, that there were significantly higher levels of the soluble form of the cellcell adhesion molecule E-cadherin in malignant ovarian

cyst fluid compared to fluid from benign ovarian cysts (Sundfeldt et al., 2001). This result was supported by another study done by Darai et al (1998), which also showed that soluble ICAM-1 and CD44 were elevated in the cyst fluid of borderline and malignant tumours compared with benign cystadenomas. Elevated concentrations of such cell-cell adhesion molecules in biological fluids in contact with neoplastic cells have also been found to be of diagnostic and prognostic use in cancers of the bladder (Jackson et al., 1993) and stomach (Matsuura et al., 1992).

Gonadotropins

Gonadotropins like follicular stimulating hormone (FSH) and luteal hormone (LH) were found to be highly expressed in the cyst fluid of malignant serous ovarian cysts, while they had very low expression or were absent in benign ovarian cysts (Kramer et al,1998, Chudecka-Glaz et al, 2003). Gonadotropins can stimulate ovarian cancer growth in cell cultures. Cell lines expressing gonadotropin receptors are also more resistant to chemotherapy (Chudecka-Glaz et al, 2003)

Proteolytic enzymes

For the dissemination of malignant cells and cancer progression, the dissolution of the basement membrane and the extracellular matrix surrounding the tumour is critical. A study by Koivunen et al reported increased concentrations of a tumour associated trypsinogen isoenzyme (TAT-2; a 28 kDa protein as determined by SDSPAGE) in the cyst fluid of malignant serous and mucinous ovarian cysts compared to benign ovarian cysts (Koivunen et al., 1990). The level of TAT-2 in cyst fluid was also found to correlate with the degree of malignancy of the tumour. Increased levels of protease inhibitor in association with cancer have also been observed, which may reflect a defence mechanism of the host against tumour invasion (Chawla et al., 1984).

Tissue polypeptide specific antigen (TPS)

Levels of TPS, a marker that is used to measure tumour cell activity in many cancers, have also been reported to be higher in cyst fluid from patients with ovarian carcinoma compared with the levels in patients who had benign cysts ($p < 0.001$) (Sedlaczek et al., 2002). However, there was no significant correlation between TPS levels and FIGO cancer stage.

Cancer antigens

A number of studies have also looked at CA-125 to examine whether its over-expression in the serum of patients with EOC is mirrored in cyst fluid. Interestingly, no correlation between CA-125 levels in cyst fluid and whether the cyst was benign or malignant was found (Sedlaczek et al., 2002). Other proteins that were found to be over-expressed in the cyst fluid of malignant epithelial ovarian cysts include CA 19-9 and carcinoembryonic antigen (CEA), although there are conflicting reports for these markers (Cherchi et al., 2002). Alterations in the plasminogen activation system have also been described in the cyst fluid of ovarian tumours (Boss et al., 2002).

Use of intracystic biomarkers in the clinical setting

The search for protein biomarkers of ovarian cancer has for many years focused primarily on serum biomarkers for the early diagnosis of the cancer. It is only fairly recently that there has been growing interest in cyst fluid protein biomarkers, which can be used for the intra-operative diagnosis of ovarian cancer. Unfortunately, none of the intra-cystic biomarkers that have so far been proposed as useful in differentiating between benign and malignant ovarian cysts are currently used in the clinical setting. In general, this is because these biomarkers have not been studied extensively enough, especially in terms of the sensitivity and specificity that can be achieved by using them to differentiate between a

benign and a malignant ovarian cyst. VEGF is one of the most promising cyst fluid biomarkers that have been reported. The study by Demirkiran et al found that by using a cut-off value of 16 ng/ml for VEGF concentration in ovarian cyst fluid, a sensitivity and specificity of 98% and 80% respectively could be achieved for the intra-operative diagnosis of EOC (Demirkiran et al., 2003). Whether, by using cyst fluid VEGF levels as an adjunct to frozen section, the accuracy of intra-operative diagnosis can be improved to what is achieved by frozen section alone needs to be studied.

The adhesion molecules seem to be plausible potential biomarkers for ovarian malignancy since these molecules play an important role in allowing malignant cells to spread locally and also to metastasize. Soluble E-cadherin, in particular, has been studied more than the other adhesion molecules, especially in terms of its clinical relevance as a cyst fluid biomarker. Using a threshold for the intracystic concentration of E-cadherin as 10,000 ng/ml, sensitivity of 69% with a positive predictive value of 100% and a negative predictive value of 92% was obtained (Darai et al., 1998). These results, although promising, need to be verified in a larger sample size than was used in this preliminary study by Darai et al (1998).

Many of the intra-cystic biomarkers reported to be of potential use in diagnosing ovarian malignancy have not been studied further in terms of their clinical relevance. Such biomarkers include a number of inflammatory cytokines e.g. IL-8, gonadotropins, proteolytic enzymes e.g. TAT-2 and TPS. Without knowing the sensitivity and specificity that can be achieved by using an appropriate cut-off concentration, it is difficult to assess whether these markers are likely to be of any use in the future in the clinical setting. Of these biomarkers, however, TAT-2 could potentially be of the most interest, as its levels in cyst fluid were found to

correlate with the degree of malignancy of the tumour (Koivunen et al., 1990). This makes TAT-2 of potential use in not only creating a high index of suspicion for malignancy and to triage for frozen section, but also as a marker for the tumour grade which may affect surgical management.

1.3.2.2 Protein markers in peripheral blood

1.3.2.2.1 CA-125

The most thoroughly assessed ovarian cancer biomarker is CA-125. The discovery of CA-125 dates back to the early 70's when attempts were made to obtain monoclonal reagents for serotherapy of patients with ovarian cancer (Order et al., 1973). In animal model studies, the intraperitoneal injection of a heteroantiserum from rabbits raised against a purified tumour-associated antigen could inhibit the growth of intraperitoneal ovarian cancer transplants in mice (Order et al., 1973). With the advent of the monoclonal technology by Kohler and Milstein in 1975 (Kohler et al., 1975), attempts were made to develop antibodies reactive with ovarian cancer cells (Knapp et al., 1977; Bast et al., 1981). After repeated injection of a human ovarian cancer cell line in mice, the hybridomas were prepared from spleen cells and the P3NS-1 plasmacytoma. The clones producing antibodies that bound to the cancer cell line were then isolated and an IgG1 antibody generated with desired specificity was designated OC-125. This antibody was found to bind the CA-125 antigens using immunohistochemical techniques, which was found to be expressed by approximately 80% of EOCs as well as by other carcinomas (Knapp et al., 1977; Bast et al., 1981). Mucinous cystadenocarcinomas were less frequently positive when compared with serous and endometrioid adenocarcinomas (Kikkawa et al., 1996).

The characterisation of CA-125 began in the mid-1980s. CA-125 was found to interact with a family of high molecular weight glycoproteins which contain multiple CA-125 antigenic determinants. It differed from classical mucins by the percentage of carbohydrate conversion and presence of both N-and O-linked carbohydrate residues (Davis et al., 1986). The biologic function of these CA-125 antigenic determinants was unknown, but studies indicated that they might be positively stained in a wide range of normal adult tissues including pleura, pericardium and peritoneum but not in the normal adult ovary (Kabawat et al., 1983). A serum CA-125 level of 35 U/ml is usually accepted as the upper limit of normal range (Bast et al., 1985). The mechanisms by which serum CA-125 levels are elevated remains obscure at present but perhaps relates to disruptions of tumour-vascular interface (Bast et al., 1985).

There are three major roles of CA-125 in the diagnosis and management of ovarian cancer (Bast et al., 1985):

1. Evaluating the therapeutic effectiveness in patients diagnosed with ovarian cancer.
2. Monitoring therapy of women known to have EOCs following cytoreductive surgery.
3. Detecting the recurrent disease during post-treatment surveillance.

The use of CA-125 as a screening tool was initiated due to the fact that 83% of patients with EOCs had a CA-125 level of more than 35 U/ml (Bast et al., 1985). This elevation of CA-125 was found in 50% of patients with stage I cancer and >90% of women with more advanced stages, though mucinous and borderline tumours were likely to be present with lower levels of CA-125 than serous cancers (Bast et al., 1985). Regular pelvic examinations and CA-125 measurements followed by radiological diagnosis on an individualised basis have

been the current practice for detection of this enigmatic condition (Mackey et al., 1995). One of the earliest attempts to use the CA-125 assay as a screening tool was reported by Jacobs et al in 1998, who evaluated 1010 apparently healthy postmenopausal women by pelvic examination and serum CA-125 measurement (Jacobs et al., 1988). Participants with abnormal findings underwent abdominal ultrasonography and those with abnormal CA-125 examinations were referred to a gynaecologist for surgical evaluation. The specificity of this assay was calculated to be 97%, which was below the estimated specificity of 99.6% necessary to keep the positive predictive value of ≥ 10%. Although current early detection protocols generally involve a combination of ultrasound and serum CA-125 levels, these criteria have met with limited success (Karlan et al., 1999). The largest randomised screening trial to date evaluating more than 20,000 women indicated a survival benefit in such women, the cancer was however undetected at an early stage and mortality rates were not significantly different between the screened and control groups of women (Jacobs et al., 1999). As a marker for screening test, the data on serum CA-125 alone are disappointing, due both to the relatively high incidence of false-positive findings and also to the fact that only 50% or fewer of patients with stage I disease have elevated CA-125 levels.

Even though high CA-125 level was observed in serum of malignant ovarian tumours, the false-positive rate of remains high in part due to its elevation in benign gynaecology conditions such as ovarian cysts, endometriosis and uterine fibroids (Fisken et al., 1993; Mackey et al., 1995; Cannistra, 2004) and conditions in other systems such as hepatic disease, renal failure and pancreatitis (Bastani et al., 1995; Devarbhavi et al., 2002). Although a combination of tumour markers could be one method to overcome this problem, there is currently no evidence that any such biomarker combination could improve the detection rate of EOC when compared with CA-125 alone. Screening with additional serum markers

including CA-199 and lysophosphatidic acid as adjuncts to CA-125 have not enhanced its diagnostic value (Xu et al., 1998; Woolas et al., 1999).

1.3.2.2.2 Other EOC-associated molecules

Although serum CA-125 levels have consistently proved to be the most useful tumour marker for evaluation and monitoring of patients with known or suspected EOC, a number of additional tumour-associated molecules have been extensively evaluated as well.

Cancer Antigens

Alpha-fetoprotein (AFP) is an oncofetal glycoprotein normally present in the fetal circulation and re-expressed in adults who have germ cell or hepatic tumours as well as in some benign medical conditions (Gillespie et al., 2000). The serum half-life of AFP is 4 to 6 days, and its levels are usually measured prior to administration of chemotherapeutic drugs (Schwartz, 1984). Although mild elevations in AFP have been observed in EOC (Chumas et al., 1984), the main clinical utility of serum AFP levels in gynaecologic oncology is in the monitoring of patients with germ cell malignancies (von Eyben, 2003). it was reported to be correlated with increasing stage of germ cell tumours, with degree of resection of primary tumour following cytoreductive surgery, and most importantly with response of residual disease to chemotherapy (von Eyben, 2003).

Carcinoembryonic antigen (CEA) is another oncofetal glycoprotein that has been extensively studied in a wide variety of neoplasms (Wahlstrom et al., 1979). Unfortunately, the role of serum CEA levels in monitoring therapy of patients with EOC remains to be established. Elevated serum CEA levels are more likely to be associated with mucinous adenocarcinomas. Some patients with mucinous

tumours show rising serum CEA levels during disease progression, but little correlation exists between clinical response to therapy and decline in CEA because the initial elevations of CEA in many of these patients are modest (Bast et al., 1984).

Cancer antigen 19-9 (CA19-9) and 153 (CA-153) like CA-125 are both monoclonal antibody-defined tumour markers. CA19-9 is a carbohydrate determinant recognised by a monoclonal antibody originally developed against a human colon carcinoma cell line (Koprowski et al., 1979). CA-153 is a breast cancer-associated antigen identified by two monoclonal antibodies, 115D8 and DF3. The first antibody reacts with the high-molecular-weight glycoprotein MAM-6 expressed by most carcinomas, whereas the second antibody recognises an antigen with a molecular-weight of 260 kDa present in a membrane-enriched fraction of metastatic breast cancer cells (Kufe et al., 1984; Hilkens et al., 1986). CA19-9 is elevated primarily in patients with carcinomas of the gastro-intestinal tract and CA-153 is recommended in the monitoring of the course and response to therapy of breast cancer (Pronk et al., 1997; Wolfrum et al., 2005). However, both of them are elevated in ovarian cancer and reported as adjunct biomarkers to CA-125 in monitoring therapy and detecting recurrence of this disease (Bast et al., 1984; Scambia et al., 1988).

Cancer antigen 72-4 (CA72-4) or tumour-associated glycoprotein 72 (TAG 72) is a mucin-like, glycosylated surface antigen found in colon, gastric, and ovarian cancer with a molecular weight greater than 1000 kDa. It is recognised by the two murine IgG monoclonal antibodies: B72.3, isolated using a metastatic breast cancer cell membrane component from liver and CC49, isolated using the LS-174-T colon cancer cell line (Colcher et al., 1981). CA72-4 is expressed by a wide range of human adenocarcinomas and is regarded as a serum marker for

the postoperative monitoring of patients with colon and gastric cancers (Klug et al., 1986; Gangopadhyay et al., 1998). As for ovarian cancer, CA72-4 is more frequently elevated in mucinous tumours (Kikkawa et al., 2006). However, there is no significant improvement in the diagnosis of ovarian cancer using combination of CA72-4 and CA-125 when compared with CA-125 alone (Negishi et al., 1993).

Lysophosphatidic acid (*LPA)*

LPA is a bioactive phospholipid with mitogenic and growth factor-like activities. It mediates a number of biological responses through activation of G protein-coupled plasma membrane receptors (LPA1, LPA2, and LPA3) (Moolenaar et al., 1997) and is involved in many biological processes, such as transient elevation of cytosolic free calcium, phosphorylation of specific cellular proteins including focal adhesion kinase, activation of mitogen-activated protein kinases, and formation of focal adhesions (Chrzanowska-Wodnicka et al., 1994; Xu et al., 1995). Plasma LPA levels may represent a potential biomarker for ovarian cancer and other gynaecologic cancers. Studies (Xu et al., 1998) showed that elevated LPA levels were detected in 90% patients with stage I and 100% patients with stage II–IV ovarian cancer. In comparison, only less than 60% patients had elevated CA-125 levels, including 2 out of 9 patients with stage I disease. In another study LPA and other lysophospholipids were found to be useful markers for diagnosis and prognostication of ovarian cancer in a controlled setting (Sutphen et al., 2004). The combination of the above mentioned lysophospholipids yielded the best discrimination between pre-operative case and control samples, with 91.1% sensitivity and 96.3% specificity (Sutphen et al., 2004).

Prostasin

Chemically, prostasin is a trypsin-like serine proteinase secreted by the prostate gland with a molecular mass of 40 kDa (Yu et al., 1994). Like the enzymatic activity of other serine proteinases, the activity of prostasin depends upon a catalytic triad of the amino acids histidine, aspartic acid, and serine (Yu et al., 1995). It was identified as a potential biomarker following discovery of its mRNA overexpression in ovarian cancer patients when compared with normal human ovarian surface epithelial cell lines using microarray technology (Mok et al., 2001). The use of a combination of prostasin and CA-125 in 37 patients with nonmucinous neoplasms and 100 control subjects resulted in improved sensitivity (92%) and specificity (94%) for detection of ovarian cancer (Mok et al., 2001).

Osteopontin (OPN)

OPN is another biomarker that has been identified by exploiting gene expression profiling techniques. It exhibited a 184-fold overexpression in pooled ovarian cancer cell lines when compared with human OSE (Wong et al., 2001). As a secreted phosphorylated glycoprotein, OPN is abundant in bone mineral matrix and accelerates bone regeneration and remodelling. It is also produced in tumour tissues and involves in the regulation and progression of many cancers by enhancing the invasive and proteolytic capabilities of tumour cells (Agrawal et al., 2002). In a study conducted by Kim and coworkers, OPN was observed to be significantly elevated (P <0.001) in sera from EOC patients when compared with healthy controls (Kim et al., 2002).

Growth factors and cytokines

Cytokines and growth factors have been observed to play important roles in the development of various kinds of neoplasms (Aaronson, 1991; Cross et al., 1991) and it has been proposed that EOC may be a cytokine propelled disease (Malik

et al., 1991). Involvement of these molecules in the tumourigenesis of ovarian cancer opens a door for researchers to assess their expression in tissue, serum, plasma and the potential roles in diagnosis, prognostication as well as in the evaluation of therapeutic effectiveness.

Several growth factors have been examined in ovarian cancer including transforming growth factor-β (TGF-β), vascular epidermal growth factor (VEGF) and inhibin (Sewell et al., 2005; Inan et al., 2006; Zeineldin et al., 2006). TGF-β is a 24 kDa glycoprotein acting as an autocrine growth inhibitory factor for normal ovarian epithelial cells. It may act in a similar fashion for some ovarian cancer cells (Bartlett et al., 1992). Therefore, the loss of the ability to produce active TGF-β or to respond to this factor may have occurred in some ovarian cancers. It was reported that patients with malignant ovarian tumours expressing TGF- β and its receptors in blood vessels had longer survival rate than those with negatively expressed tumours (Henriksen et al., 1995). The aberrant levels of TGF- β and the significant correlations between this ligand and patient survival indicate the prognostic significance of this biomarker for ovarian cancer.

VEGF is a growth factor responsible for proliferation of vascular endothelial cells and vascular permeability. It acts through two specific tyrosine-kinase receptors termed flt and KDR (Neufeld et al., 1994). This growth factor is secreted by several normal cell types as well as different tumour cells. For instance the expression of mRNA for VEGF and its receptors were detected in ovarian cancer cell lines (Boocock et al., 1995) and surgical samples from primary and metastatic ovarian carcinomas (Mattern et al., 1997; Lee et al., 2006). Moreover, elevated serum VEGF levels were observed in ovarian cancer patients (Rosen et al., 2005). It was reported in a study including 53 ovarian cancer patients that pre-operative serum VEGF levels were significantly higher in patients with

advanced neoplasm (*P* = 0.027), but not in those with early cancers (Gadducci et al., 1999). Among patients with advanced disease, VEGF levels were significantly higher in women with ascites (Gadducci et al., 1999). These data indicate that VEGF has a definite biological role in tumour progression and in the pathogenesis of ascite formation. It was also reported using multivariate analysis that elevated pre-treatment serum VEGF could serve as a independent prognostic factor since it was significantly correlated with poor disease-free survival and overall survival of ovarian cancer patients (Cooper et al., 2002; Hefler et al., 2006).

Inhibin A or B is a hetero-dimer consisting of α and either βA or βB subunits linked by disulphide bond. It is produced primarily by the gonads with an essential role in regulating pituitary FSH secretion by a negative feedback mechanism (Knight, 1996). Serum inhibin is a product from ovary and it decreases to non-detectable levels after menopause. However, mucinous carcinomas and some sex cord stromal tumours continue to produce inhibin, which provides a basis for a serum diagnostic test (Knight, 1996). Available data indicated that when used in combination with CA-125 inhibin had an increased sensitivity (95%) and specificity (95%) for detection of ovarian cancer (Robertson et al., 2002).

Various cytokines, including tumour necrosis factor- α (TNF- α), macrophage/monocyte colony stimulating factor (M-CSF), and interleukin 6 (IL-6) may also play important roles in ovarian cancer pathogenesis (Scambia et al., 1996; Chen et al., 2001; van Haaften-Day et al., 2001; Wang et al., 2005; Muthukumaran et al., 2006). In an Italian study, raised IL-6 concentrations (>6pg/ml) were detected in 50% of 114 patients with malignancies. However, the sensitivity of diagnosis using serum IL-6 was lower than that of CA-125 and the

combination of both assays showed no improvement when compared with CA-125 alone (Scambia et al., 1995). It was noteworthy that ovarian cancer patients with high IL-6 levels had a significantly worse survival outcome than those with low IL-6 levels (P = 0.0009)(Scambia et al., 1995).

Tumour necrosis factor-α (TNF-α) was originally identified as an agent that was selectively cytotoxic for some tumour cell lines (Old, 1985) and caused necrosis of certain murine tumours (Palladino et al., 1987). However, this cytokine has a number of biological activities that could promote the growth and invasive capacity of tumours (Fiers, 1991). TNF-α was found expressed by the tumour epithelium and infiltrating macrophages using in situ hybridisation and the expression levels were correlated with increasing tumour grade in serous ovarian carcinoma (Naylor et al., 1993). Others have shown that TNF-α mRNA is expressed by ovarian carcinoma as well as OSE cells, with the function to promote growth in both cell types (Takeyama et al., 1991; Wu et al., 1993).

Macrophage colony-stimulating factor (M-CSF) is a cytokine produced constitutively by normal as well as neoplastic ovarian epithelium. Many ovarian cancer cells express both M-CSF and fms, the M-CSF receptor (Ramakrishnan et al., 1989). M-CSF is a homodimeric 90 kDa glycoprotein. It has been postulated that M-CSF by stimulating macrophages could produce other cytokines, such as IL-I or IL-6 that can further stimulate tumour cell growth (Ziltener et al., 1993). Diagnostic and prognostic potential of M-CSF and its receptor was also investigated in ovarian cancer. Levels of fms transcripts were strongly associated with ovarian tumours in high histological grade and advanced clinical stage with a poor clinical outcome (Chambers et al., 1997). Moreover, elevated plasma levels of M-CSF were observed in 70-80% of patients with ovarian cancer (Kacinski et al., 1990). Combination of M-CSF with other biomarkers had

achieved a better sensitivity in discriminating malignant ovarian tumours from benign pelvic masses (Woolas et al., 1995).

Based on previous studies, it could be surmised that growth factors and cytokines could potentially act as autocrine or paracrine stimulatory factors which modify the tumour cell environment, resulting in enhanced proliferation and metastatic characteristics of ovarian cancer. The potential role of cytokines and other immunomodulatory agents in the diagnosis, prognosis and management of ovarian cancers could provide a better understanding of the gamut of these factors in the microenvironment of the tumours and the malignant behaviour of the tumour itself.

1.3.2.2.3 Multiplex platform for biomarker application

It has been suggested that the multi-parametric analyses could increase sensitivity and seem to be a logical approach for early diagnosis of ovarian cancer, although increased sensitivity is usually associated with decreased specificity (Diamandis, 2002). Important progress has been achieved by the use of the combination of CA-125 and ultrasound. The specificity of screening using CA-125 alone was improved by the addition of pelvic ultrasound as a secondary test to assess morphology of the ovary (Kupesic et al., 2002). It was further improved by combination of sequential CA-125 and pelvic ultrasound resulting in a specificity of 99.9% for detection of ovarian cancer (Jacobs et al., 1988). It was also observed in the same study that CA-125 levels in healthy women were static or decreased while levels in malignancies were significantly elevated with time. A more sophisticated approach therefore would be to interpret serial CA-125 results other than a fixed absolute cut-off level. The algorithm developed from this serial

CA-125 profile increased the sensitivity in diagnosis when compared with a single cut-off value and it improved the specificity at the same time (Jacobs et al., 1988).

Panels of protein markers were also investigated in detection of ovarian cancer. Based on cDNA microarray analysis in normal OSE and 42 ovarian cancers of different stage, grade, and histology, a panel of biomarkers have been identified as the potential candidates for the diagnosis of ovarian cancer (Lu et al., 2004). Using immunohistochemistry method, a combination of CLDN3, CA-125, and MUC1 could result in a sensitivity of 99.4% in detecting 158 cancers from normal ovarian samples, and 100% with a combination of CLDN3, CA-125, MUC1, and VEGF (Lu et al., 2004). Another study using a panel of eight different markers (CA-125, M-CSF, OVX1, LASA, CA15-3, CA72-4, CA19-9, CA54/61) achieved a better sensitivity in differentiation of malignant from benign adnexal masses (Woolas et al., 1995). The same group later demonstrated that a prototype artificial neural network developed from four markers might dramatically improve sensitivity compared to CA-125 alone (87.5% versus 68.4%) while maintaining the specificity. In addition, application of multiple markers may increase the specificity when serial cut-off values were employed (Zhang et al., 1999). In a preliminary study using a panel of five tumour markers (CA-125, HER-2/neu, urinary gonadotropin peptide, lipid-associated sialic acid, and Dianon marker 70/K), a population specific pool of markers could be obtained to improve the early diagnosis of EOC from 1,257 high-risk women (Crump et al., 2000).

Studies also demonstrated that the use of panel of multiple cytokines, which individually may not be useful clinically, might proved to be of highly diagnostic value (Gorelik et al., 2005). A novel multianalyte profiling technology was designed which allowed simultaneous measurement of multiple cytokines and growth factors. Concentrations of 24 cytokines and CA-125 were measured in

sera from patients presenting with ovarian cancer and healthy controls. Six of them including IL-6, IL-8, EGF, VEGF, monocyte chemoattractant protein-1 (MCP-1), and CA-125 demonstrated significant different serum concentrations between ovarian cancer and control groups. The classification tree analysis designed from these cytokines resulted in 84% sensitivity and 95% specificity compared with 74% sensitivity and 80% specificity using CA-125 alone. This study indicated that simultaneous testing of a panel of serum cytokines and CA-125 might provide a promising approach for ovarian cancer detection. A more recent report indicated that using three markers (IL-18, FGF-2, and CA-125) the sensitivity in detection ovarian cancer was similar compared to that of CA-125 alone. However, the specificity was significantly improved in detection of ovarian cancer (20/25 patients) compared to that from individual assessement of each marker (Le Page et al., 2006).

Although endeavours have been made in the quest for candidate biomarkers for ovarian cancer, the above mentioned proteins have not been fully evaluated with randomised clinically controlled trials or tested in a multicentre study. A more thorough investigation of these biomolecules is needed before they can be used in screening the general population. The ideal tumour marker should be a substance unique to a particular neoplasm and distinct from the normal tissue. It should also be detectable and quantifiable with consistency and reproducibility, sensitive enough to detect cancer at an early stage and be specific enough to distinguish the benign and malignant forms. In order to develop a suitable diagnostic test, several criteria are needed which includes the important characteristics such as sensitivity, specificity, and predictive values. Most clinical researchers in this area of investigation concur that in order to be acceptable in this context an ovarian cancer test must achieve a minimum PPV of 10% (Jacobs et al., 2004). Considering the low abundance of ovarian cancer, a test for ovarian

cancer is required with a view to achieve a minimum of 99.6% specificity which possess a challenge to the efforts for the appropriate identification of additional molecules to the already established pools of biomarkers.

1.4 Application of proteomics on biomarker discovery

Development of high throughput biotechnology and bioinformatics are making it possible to simultaneously analyse the entire components of proteins expressed in a particular type of cell or tissue. These advances have created unique opportunities in the field of medicine, where the results of gene and protein expression studies are expected to help identify cellular alterations associated with disease aetiology, progression, outcome, and response to therapy. As we enter into the post genomic era, development of proteomics promises to revolutionise and provide high throughput approaches for biological studies (Figure 1.3).

The term “proteomics” was coined in 1995 as global analysis of the proteins expressed in a cell, tissue or body fluid (Wasinger et al., 1995). Recent advances in proteomic profiling technology have made it possible to apply computational methods to detect changes in protein expression profiles and their association to disease processes. In the past 5 years, significant effort has been made in identifying reliable novel protein markers for early detection and prognostication of ovarian cancer using proteomics based methods. Such information will be crucial in our understanding of cancer prognosis, diagnosis and therapeutics with the ultimate goal being in the identification of potential markers for early detection and prognostication of this insidious disease.

1.4.1 Principle of mass spectrometry

The mass spectrometry determines the mass-to charge (m/z) ratio or a property related to m/z. A mass spectrum is the curve of ion abundance versus m/z,

although in most cases the x-axis is simply labelled as ‘mass’ rather than m/z (Glish et al., 2003). Mass spectrometers consist of three basic components: an ion source, a mass analyser and an ion detector (Figure 1.4). For analysis of biological samples by MS, molecules must be converted into gas phase ions using the laser energy. A mass analyser measures these ions according to their m/z ratios. The motion of such ions is then recorded based on the number of events or electrical currents created (Glish et al., 2003). This sequence of events involving proteomics is illustrated in the Figure 1.4.

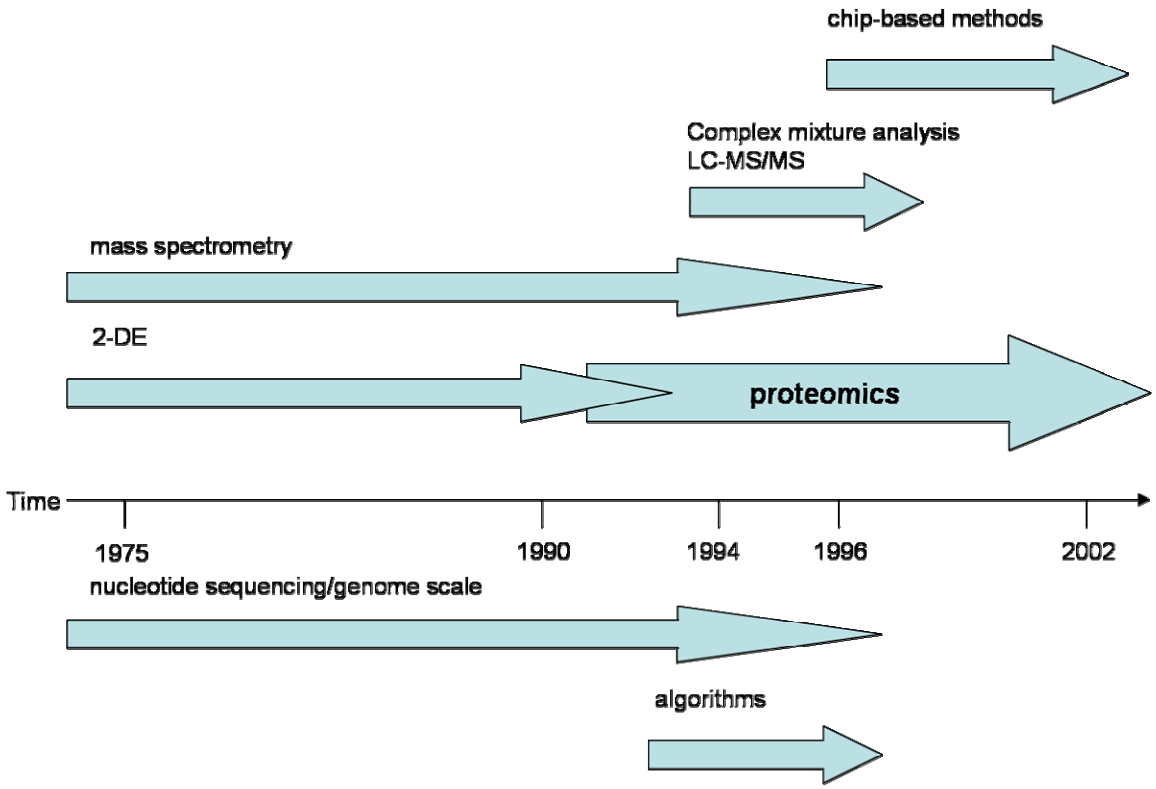


Figure 1.3 Time line indicating the development of proteomics technology.

With the advances in mass spectrometry, large nucleotide sequence dataset information, coupled to computational algorithms and bioinformatics resources resulted in the emergence of proteomics.

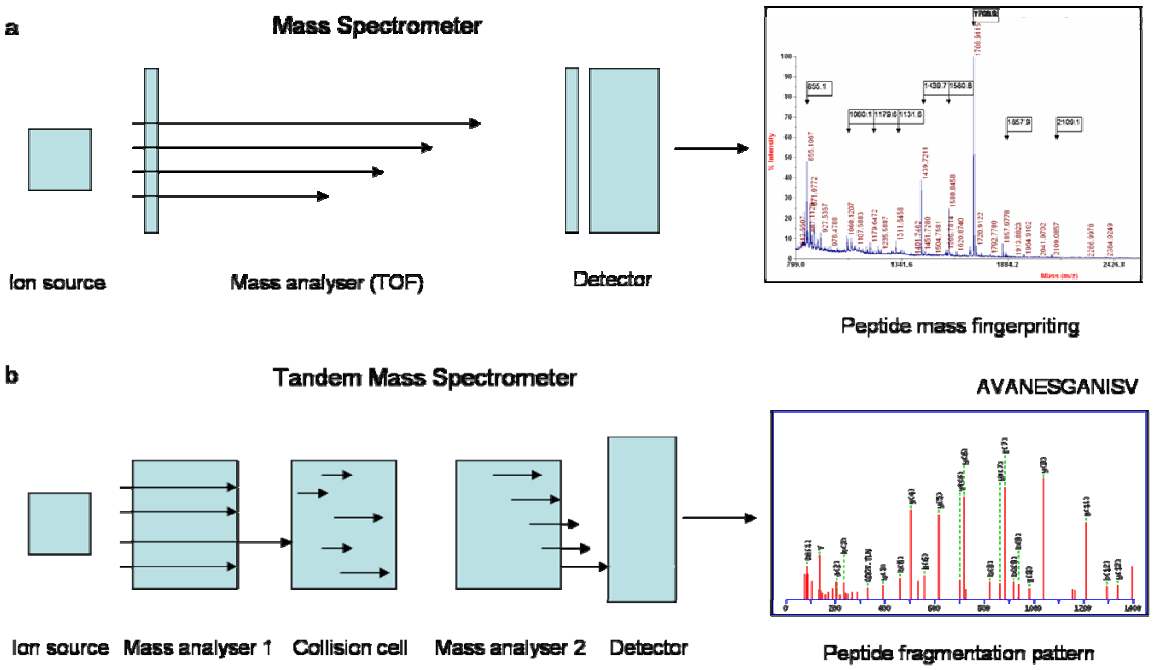


Figure 1.4 Illustration of mass spectrometer

(a) A single-stage mass spectrometer. The instrument consists of three components: an ionisation source, mass analyser and ion detector. The mass analyser shown in the figure is a time-of-flight (TOF) mass spectrometer. The mass-to-charge ratio (m/z) of ions was measured. A mass spectrum of a protein digest is shown to the right of the figure. (b) The components of one type of tandem mass spectrometer. The instrument consists of an ion source, first mass analyser, gas-phase collision cell, second mass analyser and ion detector. The first mass analyser can be used to scan and isolate a parent ion for dissociation in the collision cell. The dissociation products are then analysed in the second mass analyser. A fragmentation peptide pattern is shown to the right of the mass spectrometer.

1.4.1.1 Ionisation techniques

The electrospray ionisation (ESI) (Whitehouse et al., 1985; Fenn et al., 1989) and MALDI (Karas et al., 1988) are two primarily used ionisation techniques. These ionisation techniques are 'soft' procedure, which will not significantly change the integrity of large molecules ionised.

ESI operates at atmospheric pressure and passes a continuous stream of charged droplets in a solution-based environment when a high electric potential is set between a capillary and the inlet to a mass spectrometer. As the droplets evaporate in a drying gas or heat atmosphere, the charged droplets become free of the surrounding solvent and eventually ions are formed (Fenn et al., 1989). The introduction of microelectrospray and nanoeclectrospray (Emmett et al., 1994; Wilm et al., 1996), coupled with high performance liquid chromatography (HPLC) (McCormack et al., 1997), allows analysis of a very small amount of sample mixture. In addition, multiply charged ions which are generated by ESI could lower the m/z values in order to analyse higher molecular weight molecules and thus allow no limitations to the size of molecules subjected to analysis. Multiple protonation of peptides and proteins could further dissociate under activation with ESI. The features of this technique have been effectively utilised in proteomics research.

In the MALDI technique, however, samples are co-crystallised with a matrix and ionisation occurs with absorption of energy from the laser source. The formation of singly charged ions resulting from proton transfer from the sample to matrix is generally observed in the gaseous phase. Usually, small UV absorbing molecules such as 2, 5-dihydroxybenzoic acid and alpha-cyano-4-hydrozycinnamic are employed as matrix. Ions in MALDI are generated by

pulsed-laser beam rather than in a continuous process. In contrast to ESI, MALDI has a relatively good tolerance to salts and buffers which makes sample preparation more readily with this technique. Furthermore, ions generated are in batches coincident with a pulsed laser beam rather than a continuous mist, which allows customised MS/MS analysis (Yates, 2004). The comparison of ESI and MALDI ionisation is listed in Table1.4.

Table 1.4 Comparison of ESI and MALDI ionisation methods

ESI		MALDI	
Pros	Cons	Pros	Cons
Multiple charging give low m/z for high MW samples - use small mass spectrometer	Multiple charging can give confusing spectra	Singly charged ions Sample in solid state	Singly charged ions
Direct coupling of LC to MS for on-line separations	Low tolerance for mixtures -on-line LC separation recommended	Good throughput	Off-line analysis, not time-limited for MS/MS
	Requires good desolvation	Relative tolerance to salts and buffers	Sample preparation
Mass range up to 70kDa, Better mass accuracy via multiple charging	Intolerant to salts, Ion suppression	Mass range up to 300kDa	Relatively low resolution except at low mass

Adapted from Yates, (2004)

1.4.1.2 Mass analyser

The mass analyser is responsible for separating ions by their mass-to-charge (m/z) ratios. Mass analysers use electric and/or magnetic fields to manipulate ions in a mass-dependent manner. There are four principal types of mass analysers in use today (Table 1.5) and they could be further divided into beam analysers, in which ions enter the analysers in a beam and trapping analysers in

which ions are trapped in the magnetic field and subsequently isolated in the analyser.

Table 1.5 Comparison of performance characteristics for tandem mass spectrometers

Instrument	Resolution	Mass accuracy	MS scan rate	MS/MS scan rate
Linear Ion Traps	2–15,000	100–300 ppm	Moderate to fast	Moderate to fast
Q-QIT	2000	300-500 ppm	Moderate	Moderate
TOF-TOF	20-25,000	10-20 ppm	Fast	Slow
FTMS	50-100,00	1-2 ppm	Moderate	Moderate

ppm = parts per million

Adapted from Yates, (2004)

The most commonly used mass analysers in proteomics are TOF and Ion trap, which produce similar mass spectrum and database matching scores. A TOF spectrometer can separate ions based on their velocity, which is inversely proportional to the square root of m/z. Hence, by measuring the time the ions reach the detector, the m/z value could be estimated. The newly developed TOF/TOF instrument coupled a collision induced dissociation (CID) cell provides a better resolution and mass accuracy by obtaining MS/MS spectrum for both parent and fragment ions. The other type of spectrometer Ion-trap has three dimensional electrical fields which can trap ions inside. The mass selective mode of operation in ion trap makes the ion trajectories in an unstable condition for MS analysis. It preferentially detects a larger number of high-mass fragments than the TOF/TOF. Alternatively, the TOF/TOF detects the low-mass sequence fragments as well as internal fragments.

1.4.1.3 Database searching

The purpose of mass spectral database search is to investigate the best sequence match to the spectrum. The approaches for database search using bioinformatics methods are generally uniform. The experiment data are either peptide molecular weight digested from a protein by enzyme or MS/MS information from peptides subjected to further fragmentation. The data are then compared with the theoretical peptide mass or ion mass value in a sequence database. The corresponding mass values are counted and scored in such a way that the best matched protein is identified (Steen et al., 2004; Figure 1.5). For the unknown proteins which are not in the database, identification is performed using the closest homology in the related mammalian species. Four basic algorithms have been developed in the database searching, namely the descriptive, interpretative, stochastic and probability-based modelling. Descriptive models use mathematical methods such as correlation analysis to evaluate the quality of the peptide fragments in the spectrometer and SEQUEST is an example using a two-tiered scoring system to assess the quality of the peptide spectrum from a database (Sadygov et al., 2004). Interpretative methods including that of Peptide Search interpret the sequence from mass spectrum using probabilities or correlation analysis (Mann et al., 1994). Stochastic approaches such as SCOPE, one of the earliest algorithms in the category, create likelihood of a correct match based on probability models for the generation of tandem mass spectra and the fragmentation of peptides (Bafna et al., 2001). Statistical and probability models are used to determine the peptide identification based on empirically generated fragment probabilities (Perkins et al., 1999). Mascot is one of the most commonly used methods incorporating the probability-based scoring. There are several advantages for the probability-based scoring system. Firstly, it is useful to avoid false positive results by a

simple cut-off score. Secondly, comparison with other scoring methods is available such as sequence homology. Thirdly, the searching parameters can be readily adjusted by iteration (Perkins et al., 1999).

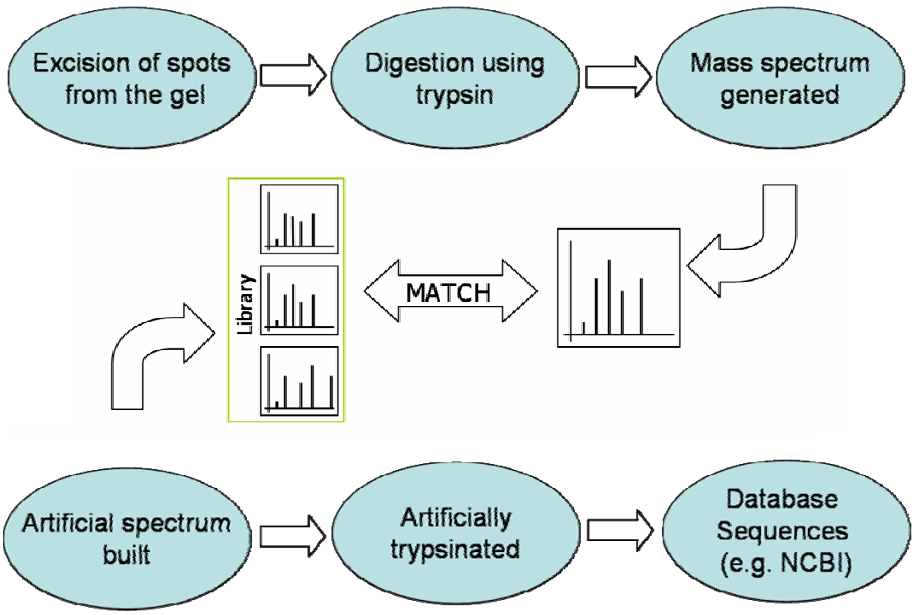


Figure 1.5 Diagram indicating the database searching for protein identification.

Tryptic digested peptides are subjected to mass spectrometry and the mass spectrum from the protein using enzyme or fragmented MS/MS information are compared with the theoretical peptide mass or ion mass value in a sequence database. The corresponding mass values are counted and scored so that the best matched protein is identified.

1.4.2 Approaches for biomarker discovery

The standard proteomic approach for biomarker research comprises protein isolation from clinical samples (tissues or biological fluids), digestion with proteases such as trypsin, separation of the resulting mixtures by gel based or solution based techniques, and identification of potential targets using MS based methods. The various techniques include 2-DE, two dimensional differential in gel electrophoresis (2-D-DIGE), SELDI-TOF, protein microarrays, MudPIT and

ESI/MALDI TOF-MS for qualitative and quantitative determination of the biomarkers (Figure 1.6).

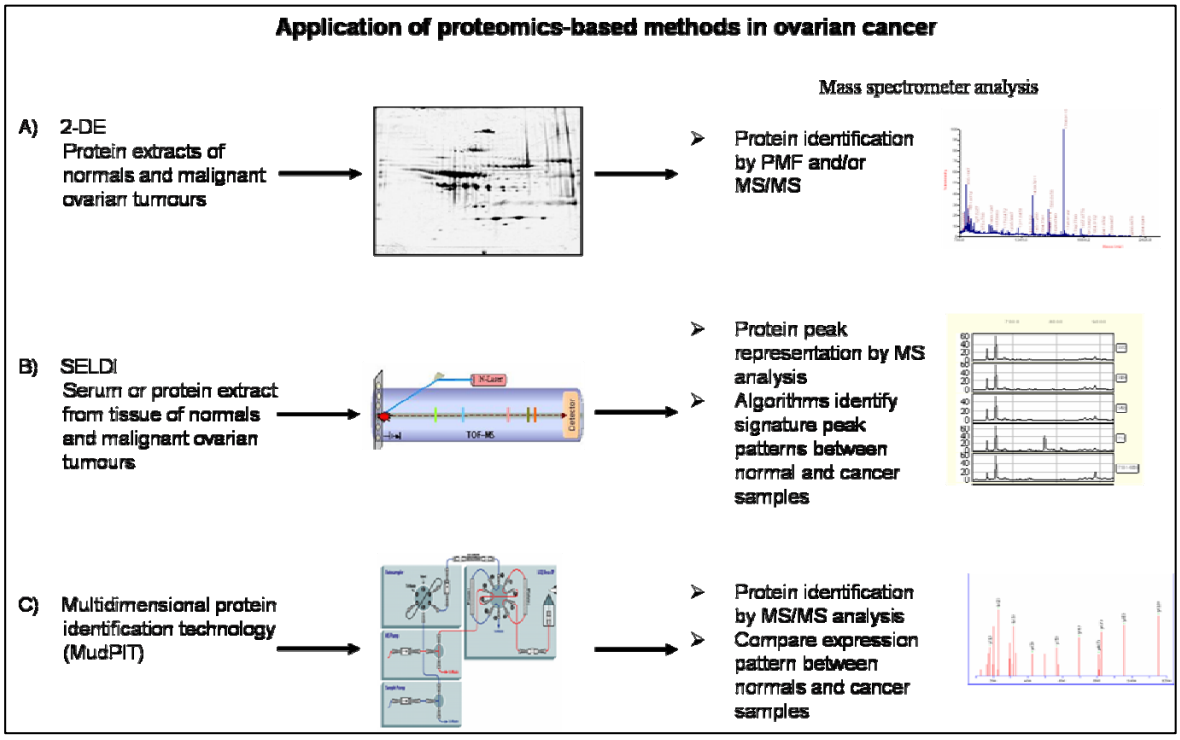


Figure 1.6 Proteomics based methods for biomarker identification

1.4.2.1 2-DE based research

1.4.2.1.1 Technical consideration of 2-DE

Conventional 2-DE using IPG strips and SDS-PAGE, combined with MS analysis has been widely utilised since the 1970s (Ballal et al., 1973). 2-DE comprising of isoelectric focusing (IEF) and SDS-PAGE enables the separation of complex protein mixtures according to p*I*, molecular weight, solubility, and relative abundance. Depending on the gel size and the pH gradient used, 2-DE can resolve more than 5000 proteins simultaneously, and can detect 1 nanogram of protein per spot (Gorg et al., 2004). Moreover, it generates a map of intact

proteins, which indicates the changes of protein expression levels. This is in contrast to liquid chromatography mass spectrometry (LC-MS/MS) based methods for peptide analyses in which molecular weight and p*I* information are not available. One of the greatest strengths of 2-DE is its capability to study proteins that have PTM including phosphorylation, glycosylation or even limited proteolysis (Table 1.6). These PTM can be identified by their specific location in 2-DE gels since they appear as distinct spot trains in the horizontal and/or vertical axis of the gels. Moreover, 2-DE not only provides information on protein modifications and/or changes in their expression levels, but also permits the isolation of proteins in milligram amounts for further structural analyses by MALDI TOF-TOF/MS, ESI-MS or Edman microsequencing (Gorg et al., 2004). In this context, 2-DE has been utilised to identify proteins that are differentially expressed between normal and tumour tissues in various malignancies, such as cancers of the cervix, stomach, pancreas, head and neck, and vagina (Ahmed et al., 2004; Chen et al., 2004a; Chen et al., 2004b; Fountoulakis et al., 2004; Hellman et al., 2004; Shen et al., 2004).

1.4.2.1.2 2-DE in ovarian cancer

Extensive 2-DE studies have been carried out using ovarian cancer cells, serum and plasma proteins. A recent report of serum proteome in 38 ovarian cancer patients at different pathological grades compared with healthy women indicated that six upregulated protein spots occurred in all groups of ovarian cancer patients which were subsequently identified as isoforms of haptoglobin-1 precursor, a liver glycoprotein present in human serum (Ahmed et al., 2004). In this study, the high abundance proteins (e.g. immunoglobulin and albumin) were removed using a mixture of Affigel-Blue and protein A (5:1) in order to recover possible biomarkers present in low concentrations. It was later demonstrated

that this protein could be secreted by ovarian cancer as the strong expression of it was observed in cytoplasma of the cancer cells. In a follow-up study from the same group, IPG strips of a narrow range (pH 5.5–6.7) for the first dimension were used to improve the resolution of the spots in this region (Ahmed et al., 2005). It was observed that haptoglobin expression in sera of cancer patients decreased following chemotherapy (six cycles of taxol/carboplatin). Changes in serum expression of haptoglobin also correlated with that of CA-125 levels before and after chemotherapy. This data indicates that haptoglobin may serve as an additional biomarker in the monitoring of therapeutic intervention in women with ovarian cancer.

Plasma has also been used in the search for potential protein biomarkers. In a recent publication (Lomnytska et al., 2006), plasma proteomic profiles from ovarian cancer patients were compared to those from healthy women using 2-DE. A cohort of 39 ovarian cancer patients and 31 healthy individuals were studied. Truncated forms of cytosolic serine hydroxymethyl transferase (cSHMT), T-box transcription factor 3 (Tbx3) and utrophin were aberrantly expressed in samples from cancer patients when compared with those from healthy women (Lomnytska et al., 2006). Such studies have indicated that increased expression of the three polypeptides in plasma samples from EOC could be used as components of a multi-parameter monitoring scheme of this disease.

Primary and secondary ovarian tumour cell lines are other targets for biomarker identification as well as studying aetiological factors in the malignant transformation of the ovarian surface epithelium (OSE) to ovarian carcinoma. Using platinum sensitive and resistant ovarian cancer cell lines, researchers were able to investigate the protein signatures involved in chemoresistance (Yan et al., 2007). Reproducible and consistent deregulated protein spots on 2-DE were

identified including annexin A3, destrin, cofilin 1. These proteins demonstrated distinct functional roles in the regulation of platinum resistance and therefore use of these protein signatures in matching the right therapeutic regimen in the appropriate patient could lead to better prognosis.

Several studies have utilised a proteomic approach based on 2-DE and MS to investigate the pattern of protein expression associated with a predisposition to develop ovarian cancer (He et al., 2005; Smith-Beckerman et al., 2005). Protein profiles of ovarian surface epithelium (OSE) from women with a history of familial ovarian cancer (FH-OSE), i.e., at least two first-degree relatives with such cancer and/or testing positive for BRCA1 mutations, were compared to the OSE protein profiles from normal women (NFH-OSE). The studies illustrated differentially expressed proteins in the FH-OSE cells, in which increased expression of mesoderm induction early response gene and several chaperone proteins including BiP and GRP94 were observed (He et al., 2005; Smith-Beckerman et al., 2005). The simultaneous analysis of these proteins may have useful diagnostic and prognostic value in the high-risk women.

However, the 2-DE procedure is limited by the heterogeneous cell types of the tumour tissues. The contamination from surrounding tissue represents a significant obstacle in the search for biomarkers, especially for early-stage ovarian cancers. Recently the invention of Laser Capture Microdissection (LCM) has greatly improved the identification of diagnostic and prognostic markers due to its ability to produce pure cell populations from the surrounding tissues. LCM was firstly introduced in 1996, when it provided a more easy, precise and efficient method of tissue microdissection compared to the previous techniques (Kovach et al., 1991; Shibata et al., 1992; Emmert-Buck et al., 1996). Incorporating LCM into proteomics study could result in enrichment of candidate protein markers as

observed in the profiles of cervical, oesophageal and prostate epithelial cells (Banks et al., 1999; Emmert-Buck et al., 2000; Ornstein et al., 2000). In the report using LCM on ovarian cancer, microdissected cells from 3 patients with invasive ovarian cancer revealed thirteen spots that were uniquely present in all three invasive ovarian cancers while it was absent or under-expressed in two cases with low malignant potential (Jones et al., 2002). Mass spectrometry identification coupled with Western blotting analysis indicated three possible diagnostic markers, the 52 kDa FK506 binding protein, Rho G-protein dissociation inhibitor (RhoGDI) and glyoxalase I were specifically expressed by ovarian cancer cells (Jones et al., 2002). However, questions regarding the use of LCM to simplify comparative analyses of EOC cells in a bid to reduce false positives between normal and diseased samples require further investigations.

The new benchmark for protein analysis developed from 2-DE is 2-D DIGE. This technique was originally developed by Minden and coworkers (Unlu et al., 1997). Proteins from cancer cases and controls are differentially labelled with fluorescent dyes Cy3 and Cy5 and the mixed proteins are separated in the same 2-DE. The gel is then scanned to generate a map for the two groups of samples, and differences in intensities for a given spot can be compared (Unlu et al., 1997). Due to the fact that the same protein from different samples is run simultaneously in the same gel, the reproducibility is significantly improved compared with conventional 2-DE (Unlu et al., 1997). The 2-D DIGE method has helped in the search for biomarkers in human cancers such as oesophageal, colon, hepatocellular and pancreatic cancers (Zhou et al., 2002; Friedman et al., 2004; Fujii et al., 2005; Yu et al., 2005). This technique has also been applied to identify potential diagnostic biomarkers and therapeutic targets for clear cell adenocarcinoma, the highly malignant subtype of ovarian cancer. In the study described by Morita and colleagues, eighteen up-regulated and thirty-one down-

regulated spots were observed in two clear cell adenocarcinoma cell lines when compared with those from mucinous adenocarcinoma cell lines. The proteins identified included annexin, purine nucleoside phosphorylase and prohibitin, which were implicated in various functions of carcinogenetic process such as cell cycle regulation, transcription and cell adhesion (Morita et al., 2006). The results of this study indicate the potential for proteomic approach to identify these disease-associated proteins, which may eventually serve as diagnostic markers for clear cell adenocarcinoma.

1.4.2.2 SELDI-TOF

1.4.2.2.1 Technical consideration of SELDI-TOF

SELDI-TOF mass spectrometry is a high-throughput, affinity-based MS technique. It represents a novel proteomic approach for the exploration and measurement of cancer-associated biomarkers and proteomic patterns in biological tissues and body fluids (Merchant et al., 2000). Crude protein extracts are spotted on a specific protein chip surface with definite protein chemistry where selective adsorption takes place, resulting in the binding of the specific proteins (Merchant et al., 2000).

The distinct advantages of SELDI-TOF is that it requires only tiny amounts of sample (5-10 µg) making it ideal for limited quantity of samples as evidenced in early stage cancers (Melle et al., 2005) (Table 1.6). The system uses chip arrays with different chemical properties to capture groups of proteins that are distinctly present in different samples. The commonly used ProteinChips include normal phase (NP20), reverse phase (H4), strong anion-exchange (SAX2), weak cation-exchange (WCX2), and immobilised affinity capture (IMAC3) chips. NP20 chips

generally bind to hydrophilic proteins while the H4 chips are suitable for their hydrophobic counterparts. The SAX2 and WCX2 chips are used to analyse proteins that have negative and positive charges on the surface, respectively. IMAC3 chips are used to capture proteins that bind divalent cationic metals such as nickel, copper and zinc (ProteinChip System Users Guide, Ciphergen). SELDI-TOF technology provides very good resolution of small proteins and peptides, as well as low-abundance proteins or proteins with extreme isoelectric points, and thus may complement 2-DE analysis.

1.4.2.2.2 SELDI-TOF in ovarian cancer

SELDI-TOF technology provides complicated patterns of mass spectrometry peaks, which could be eluted and identified individually with conventional gel-based techniques (Moshkovskii et al., 2005). Alternatively, the patterns themselves could serve as diagnostic markers on the basis of distinct protein profiles. With the aid of bioinformatics data-mining systems a unique combination of mass spectra patterns are generated (Figure 1.7). The data thus obtained could be used to discriminate disease states from normal controls (Figure 1.7). The first study combining the mass spectrometry patterns and bioinformatics recognition algorithms applied this method to the early diagnosis of ovarian cancer (Petricoin et al., 2002). In this study, a discriminatory mass spectrum pattern derived from sera of women diagnosed with ovarian cancer or from healthy women was developed from a training set of mass spectra. The pattern was then applied to another set of blinded samples from both ovarian cancer and normal cases. The power of this diagnostic test was observed to produce 100% sensitivity and 95% specificity in the detection of ovarian cancers. Interestingly, this technique seemed to possess the ability to detect patients with

early stage cancer, thus increasing the chances of early diagnosis for this insidious disease (Petricoin et al., 2002). Protein pattern profiling has also been widely used to differentiate other cancers such as breast, prostate, and liver cancer from corresponding controls (Adam et al., 2002; Li et al., 2002; Poon et al., 2003). However, the data published by Petricoin's group was re-examined by other investigators who reported that the peptide fingerprinting may have represented experimental artifacts instead of real biological differences (Erika, 2004). Due to the fact that overwhelming disputes occurred using this high throughput technology, further studies are warranted to improve the potential for identifying such malignancies which could be eventually used to screen the general population (Erika, 2004).

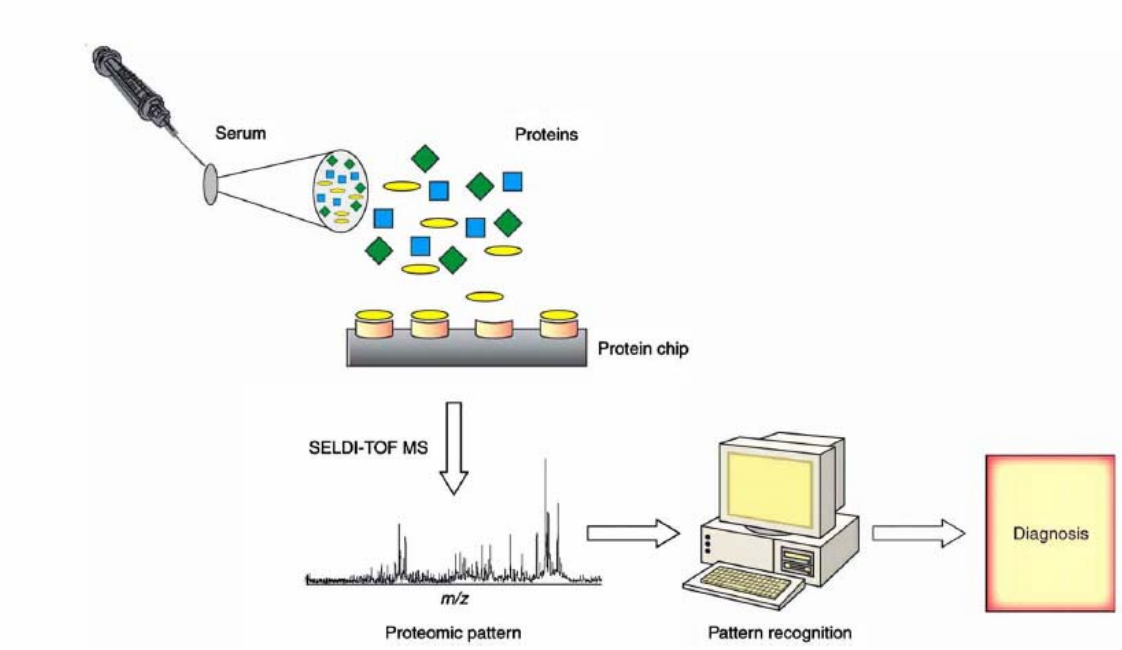


Figure 1.7 Disease diagnostics using proteomic patterns

Patents' samples are applied to a protein chip, which is made up of a specific chromatographic surface. After several washing steps and the application of an EAM, the proteins that are retained on the surface of the chip are analysed using mass spectrometry and sophisticated bioinformatics software. Specific patterns for disease versus healthy controls are generated and used to diagnose the source of the biological sample. Adapted from Rapkiewicz et al., (2004)

However, the multi-parametric analyses of Petricoin and colleagues are a logical approach to protein fingerprinting. It is generally accepted that a single biomarker may not possess the potential for the early diagnosis of ovarian and other malignancies (Zhang et al., 1999; van Haaften-Day et al., 2001; Zhang et al., 2004). Efforts should therefore be focused on the identification of a panel of novel biomarkers which are synthesised and secreted by the ovarian tumour cells. In this regard, SELDI-TOF is one of the more appropriate quantitative techniques to enable such analytical approaches required to identify specific proteins which could discriminate EOCs from normals.

A few single and multiple disease-related protein biomarkers for the diagnosis of ovarian cancer have already been identified from differential SELDI-TOF protein patterns. For example, a novel molecule—serum amyloid A1 (SAA1) was identified from SELDI-TOF protein profiles of serum obtained from 27 women presenting with EOC (Moshkovskii et al., 2005). Certain panels of protein markers have also been identified recently using a similar approach. Kozak *et al.* (2003) reported the identification of four ovarian cancer biomarker panels including transthyretin, beta-haemoglobin, apolipoprotein AI and transferrin from SELDI-TOF protein peaks. This panel demonstrated improvement in the detection rate of early stage ovarian tumours (ROC 0.933) when compared with cancer antigen CA-125 alone (ROC 0.833) (Kozak et al., 2003). More recently, Zhang et al. (2004) conducted a multicentre case-control study of serum proteomics to eliminate possible bias by cross comparison and independent validation. These investigators identified three specific biomarkers including apolipoprotein A1, a truncated form of transthyretin and a cleavage fragment of inter-trypsin inhibitor heavy chain H4. A multivariate model combining the three biomarkers and CA-125 was constructed and the sensitivity in detecting early stage invasive EOC was 74% compared with that of CA-125 alone (65%) at a

matched specificity of 97% (Zhang et al., 2004). These results suggest that the biomarkers derived from SELDI-TOF technology may add to the list of potential markers which may have the ability of detection and prognostication for ovarian cancer in the general population.

1.4.2.3 Protein microarray analysis

1.4.2.3.1 Technical consideration

Microarray analysis has a principal advantage of being a high throughput technology and offers greater precision than conventional blotting techniques (Schena et al., 1995). Such arrays possess the advantage of high-density microscopic array elements, planar glass substrates, low reaction volumes, multicolour fluorescent labelling, high binding specificity, high-speed instrumentation for manufacture and detection, and sophisticated software for data analysis and modelling (Stears et al., 2003). The development of antibody-based protein microarray technologies represents a great advancement over the conventional ELISA method (Espejo et al., 2002; Templin et al., 2002). Although the protein microarray technologies are limited by their requirement for highly specific, high-affinity antibodies as well as sensitive detection and signal amplification systems, they possess the advantages of being excellent means for simultaneous analyses of hundreds of analytes in a wide variety of disease conditions (Table 1.6) (Espejo et al., 2002; Templin et al., 2002). Microarray analysis is therefore a fundamental technology with broad applications in genetic screening, diagnostics and therapeutic assessment.

1.4.2.3.2 Protein microarray in ovarian cancer

Recently, protein microarrays have been widely utilised for identification of potential protein biomarkers for cancers (Miller et al., 2003; Huang et al., 2004; Hudelist et al., 2004; Sanchez-Carbayo et al., 2006). Using this method, a recent study reported that four out of 169 candidate protein biomarkers including leptin, prolactin, OPN and insulin-like growth factor-II were consistently deregulated in serum from patients with EOCs (Mor et al., 2005). Using a combination of these four protein markers, this study demonstrated a sensitivity of 95%, specificity of 95%, positive predictive value of 95% and negative predictive value of 94% in the diagnosis of EOCs (Mor et al., 2005). It is noteworthy that the biomarkers identified in this study were well verified by other researchers (Mok et al., 2001; Kim et al., 2002; Lu et al., 2006). In another study, Davidson and colleagues obtained cancer effusions to evaluate the biochemical status in such cancers in a bid to search biomarkers of prognostic or therapeutic significance. Using a new lysate protein array, they found that proteins involved in signal transduction and transcription regulation were deregulated in effusions from cancer patients when compared with controls (Davidson et al., 2006). Analysis of multiple serum biomarkers using a protein microarray technology appears to be a more promising approach for the development of diagnostic assays for ovarian cancer.

1.4.2.4 MudPIT

1.4.2.4.1 Technical consideration

Gel-based proteomics strategies are rapidly being replaced by techniques that involve peptide separation using high efficient nano-column liquid chromatography techniques linked to a mass spectrometer. In particular,

multidimensional protein identification technology now allows the simultaneous "shotgun" analysis of hundreds of proteins in a single experiment (Washburn et al., 2002; Wu et al., 2002). There are several advantages of MudPIT. Firstly, it avoids the tedious process of 2-DE and can run in a fully automated system. Secondly, MudPIT can identify less abundant proteins from protein mixtures just by one or two good quality MS/MS spectra. Thirdly, it is a more suitable technique for the study of hydrophobic proteins when compared with 2-DE (Link et al., 1999; Washburn et al., 2001). However, the clinical implication of these high-throughput methods has not been well defined (Hanash, 2003), in part due to problems associated with the reproducibility of the LC-MS method (Wang et al., 2003; Washburn et al., 2003). Moreover, the limited number of samples that can be analysed in reality result in difficulties in obtaining clinically relevant information from a large number of samples (Boguski et al., 2003) (Table 1.6).

MudPIT separates peptides using two-dimensional liquid chromatography. This technique requires columns consisting of strong cation exchange (SCX) and reversed phase (RP) material inside fused silica capillaries (Washburn et al., 2001). The chromatography proceeds in cycles, each comprising of an increase in salt concentration to "bump" peptides off the SCX, followed by a gradient of increasing hydrophobicity, to progressively elute peptides from the RP into the ion source (Link et al., 1999). All peptides eluted from the nano-separation column are ionised and then subjected to MS/MS analysis. Protein identity can be obtained by searching the MS/MS spectra against an appropriate database such as NCBI, Swissprot or the International Protein Index (IPI) human protein database (Link et al., 1999).

MudPIT has been successfully applied in the quantitative comparison of protein expression (Steen et al., 2004). In most circumstances, it is not only the identity

of the peptide that is of interest, but also its quantity in samples of different status. Unfortunately, the intensity of the signal of a peptide ion does not directly indicate the amount of protein present (Gygi et al., 1999). There are generally two kinds of quantification methods in MudPIT: absolute quantification and relative quantification. Absolute quantification is a laborious, but precise way of quantification by incorporating isotopically labelled peptides as 'internal standards'. The internal standards are to be mixed with the sample before digestion and loading onto the column. Absolute quantification has been applied in small-molecule MS as well as in proteomic studies of a larger scale (Barr et al., 1996; Gerber et al., 2003). On the other hand, relative quantification using stable isotopes is the accurate way of measuring the relative amounts of a protein present in two samples. The only difference in the several forms of molecules results from the substitution of the stable isotopes and there will only be a mass difference between them. Therefore, the ratio of the two peaks, which can be determined quite accurately indicates the relative amounts of a protein that is present in the two subjects (Gygi et al., 1999; Ross et al., 2004). Isotope-coded Affinity Tags (ICAT) technology is one of the widely used relative quantification methods. Originally described by Gygi *et al.* (Gygi et al., 1999), this technology allows the systematic study of the proteome and can be used to measure the abundance of particular proteins in samples from cancer versus healthy controls (Li et al., 2004; Pawlik et al., 2006).

1.4.2.4.2 Application of MudPIT

The power of MudPIT for protein identification was first shown in the analysis of total cell lysate of *Saccharomyces cerevisia* (Washburn et al., 2001). This study established that MudPIT is a powerful and unbiased method in protein identification, since large or small proteins, of high or low abundance, or

extremes in p*I* were identified with equal sensitivity. There have been several studies in cancer proteomics using the MudPIT method, but the samples used were limited due to constraints of cost and time (Chen et al., 2006). Using MudPIT technique coupled with ICAT labelling, Stewart and collaborators (Stewart et al., 2006) investigated the proteins associated with cisplatin resistance in ovarian cancer cells. The huge number of novel identified proteins involving in different metabolic pathways may confer novel insights into the mechanisms of cisplatin resistance and allow researchers to generate new strategies in chemotherapy.

The advent of proteomic technologies has provided clinician-scientists with a powerful analytical tool to investigate human diseases. The application of proteomics in studying ovarian cancer, however, is still in its early stage and remains challenging. These technologies are able to provide important information, and will have an increasing role in biomarker identification and validation in EOCs. Due to the pros and cons in each proteomics technique, only single proteomics-based method obviously limits the chances in searching suitable biomarkers which could have been identified by alternative ways. Combination of different methods therefore opens new avenues in biomarker identification for ovarian cancer. With the increased sensitivity of mass spectrometers and vigilant bioinformatics pattern-recognition tools, critical biomarker panels to be used in screening for this insidious cancer in women will undoubtedly be identified.

Table 1.6 Comparison of proteomics technology in clinical application

	2-DE	SELDI-TOF	Protein microarray	MudPIT
Advantages	High throughput, quantitative and PTM analysis	High throughput, Robust	High throughput, very robust, quantitative analysis	High throughput, very robust, quantitative analysis
Drawbacks	Laborious and time consuming, difficulty in reproducibility	Difficulty in reproducibility, diminishing sensitivity at higher molecular weight	Cost and availability of machine, limited by the availability of antibodies	Cost and availability of machine

1.5 Experiment aims and hypotheses

Ovarian Cancer is one of the most lethal gynaecologic malignancies with a long asymptomatic course often presenting at an advanced stage at diagnosis. Despite advances in surgery and chemotherapy, the overall 5-year survival rate is about 50% (Kristensen et al., 1997). Although current detection and prognostication protocols generally involve a combination of regular pelvic examinations and CA-125 measurements followed by radiological diagnosis, these criteria have met with limited success.

The search for protein biomarkers of ovarian cancer has for many years focussed primarily on serum biomarkers for the diagnosis and prognostication of the cancer. However, the cyst fluid represents an ideal source for biomarker identification in that it forms a continuous compartment surrounded by an epithelial cyst wall and the protein composition changes occurring in EOC cells will be reflected in the cyst fluid (Demirkiran et al., 2003). Hence, studies involving cyst fluid proteins are of potential significance in the identification of target markers.

The present study aims to identify useful diagnostic and/or prognostic protein markers with the potential use of cyst fluid as a model. Moreover, an intra-operative cyst fluid determination based on the differentially expressed protein levels with a specific cut-off value is developed to identify women who may require further pathological assessment with frozen section and hence, a more complex operative procedure by a cancer surgeon as opposed to a general gynaecologist.

Considering the pros and cons in each proteomics technique, the combination of two proteomics-based methods are utilised in this study in searching complimentary biomarkers. The investigations were conducted in three stages: (a) to identify candidate biomarkers by combining various proteomics-based methods including SELDI-TOF, 2-DE, MALDI-TOF, and traditional immunological methods. (b) to evaluate diagnostic and/or prognostic potential of candidate biomarkers in clinical settings and (c) to develop a portable diagnostic kit to detect EOC intra-operatively using cyst fluid.

Accordingly, the main objectives and hypotheses of these projects are as follows:

Objectives:

1. To identify a series of disease related protein markers by comparing the protein difference in cyst fluid between benign and malignant ovarian tumours using a combination of SELDI-TOF, 2-DE based proteomics methods and conventional immunological techniques.
2. To evaluate the pre-operative diagnostic and/or prognostic values of candidate protein markers in serum from patients presenting with EOC.
3. To develop a simple, portable and fast intra-operative diagnostic kit using cyst fluid as a complementary diagnostic procedure to the use of frozen section technique to differentiate malignant from benign ovarian tumours.

Hypotheses:

1. Differential disease related proteins may be present in cyst fluid from benign and malignant ovarian tumours and could be identified using a combination of SELDI-TOF, 2-DE and conventional immunological techniques.

2. Similar protein patterns may also be observed in sera from benign and malignant ovarian tumours and they may have potential roles as diagnostic and/or prognostic markers.

3. An intra-operative diagnostic assay based on the differentially expressed protein levels in cyst fluid may be developed to enable the differentiation between malignant and benign ovarian tumours at the time of surgery.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Human samples

2.1.1.1 Ethical approval for use of human samples

Cyst fluid, blood and tissue collection for this research was approved by the National Healthcare Group Domain Specific Review Board, National University Hospital, Singapore (DSRB Ref: D/00/856). All patients participating in this study gave written informed consent for the collection and use of human tissues and fluid samples.

2.1.1.2 Cyst fluid samples

Women aged 20 to 72 years with benign tumours (n=47), borderline tumours (n=10), early stage (n=13) and late stage (n=20) EOCs were enrolled for open surgical or laparoscopic treatment in the Department of Obstetrics & Gynaecology, National University Hospital, Singapore. The histologic type of ovarian cancer was classified as defined by FIGO staging (1986). Fluid samples from benign and malignant ovarian cysts were collected during surgery without intra-operative spillage. The cyst fluids were then centrifuged at 1500 g for 10 min at 4 °C and the supernatants were divided into aliquots of 1 ml each and snap frozen in liquid nitrogen for storage at -80 °C until analysis.

2.1.1.3 Peripheral blood

Peripheral blood samples (n=126) were collected by venepuncture from patients presenting with epithelial ovarian tumours at the time of diagnosis or prior to the operative procedure. Peripheral blood samples were also collected from healthy volunteers without any evidence of tumours (n=10).

2.1.1.4 Tissue samples

Tissue samples were collected intra-operatively from patients presenting with benign and malignant epithelial ovarian tumours. Samples were rinsed using sterile saline and aliquots were snap frozen in liquid nitrogen and stored at -80 °C until analysis.

2.1.1.5 Cell lines

Ovarian carcinoma cell lines TOV-112D, SKOV-3 and Caov-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.1.2 Antibodies, reagents, media, solutions and kits

All antibodies, reagents, solutions and kits used in the experiments are listed below.

2.1.2.1 Antibodies

Polyclonal rabbit anti-human haptoglobin and ceruloplasmin antibodies (Dakocytomation, Glostrup, Denmark); polyclonal goat anti-human haptoglobin

antibody (Biodesign, Saco, ME, USA); horse radish peroxidase (HRP) labelled anti-rabbit secondary antibody (Pierce Biotechnology, Rockford, IL, USA); biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA); Polyclonal rabbit antibody against haptoglobin α-subunit was generated using the following amino acid sequence, CKNYYKLRTQGDGVY (BioGenes, Berlin, Germany).

2.1.2.2 Reagents

Methanol, ethanol, acetone, chloroform, acetic acid, formic acid, hydrochloric acid, citrate, formaldehyde, isopentane (Merck, Darmstadt, Germany); dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), glycerol, bromophenol blue, thiourea, potassium ferricyanide, sodium thiosulphate, HPLC grade acetonitrile, triton X-100, ammonium sulphate, purified human haptoglobin, penicillin and streptomycin, haematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA); N,N,N',N'-tetramethylethylenediamine (TEMED), 30% acrylamide/bis solution 19:1 (5% C), SDS, Bradford assay dye reagent, ammonium persulphate, carrier ampholytes, overlay agarose, CHAPS, iodoacetamide (IAA), ethidium bromide, precision plus protein all blue® standards, mineral oil (Bio-Rad Laboratories, Hercules, CA, USA); modified sequencing grade trypsin, 6X DNA loading buffer, 100bp DNA ladder (Promega, Southampton, UK); urea, tris base (Merck, Whitehouse Station, NJ, USA); ethylenediaminetetraacetic acid (EDTA), trypsin (GIBCO, Invitrogen, Carlsbad, CA, USA); Tween 20; complete Mini® protease inhibitor cocktail (Roche diagnostics, Mannheim, Germany); dithiothreitol (DTT; Sigma-Aldrich, Oakville, Canada); 4700 proteomics analyser calibration mixture (des-Arg-Bradykinin, Angiotensin I, Glu-Fibrinopeptide B, angiotensin I and adrenocorticotropic hormone (ACTH) (1-17 clip), ACTH (18-39 clip) and ACTH (7-38 clip)) (Applied Biosystems, Framigham, MA, USA); ultra v block reagent

(BioGen Medical Instruments Inc, Turkey); non fat dry milk (Santa Cruz Biotechnology, Santa Cruz, CA, USA); alkaline phosphatase streptavidin (Vector Laboratories, Burlingame, CA, USA); fetal calf serum (Hyclone, Utah, USA).

2.1.2.3 Water and Solutions

Pure water (Milli-Q system, 18 MΩ/cm at 25 °C, with removal of organics) (Millipore, Bedford, MA, USA); 20 mM, 50 mM and 100 mM ammonium bicarbonate solution, 1x phosphate-buffered saline (PBS), 5x SDS/glycine electrophoresis buffer (15.1 g tris base, 72 g glycine and 5 g SDS, NUMI, NUS); 5 x sample loading buffer (pH 6.8; 0.313 M Tris-HCl, 10%SDS, 0.05% bromophenol blue, 50% glycerol, 0.5 M DTT) coomassie blue destaining solution (5% methanol, 7% acetic acid and 88% water); sinapinic acid solution (50% acetonitrile, 0.5% TFA); transfer buffer for Western blotting [pH 8.3; 25mM tris base; 150 mM glycine; 10%(v/v) methanol]; TBST buffer (pH 7.5; tris base; 500 mM NaCl; 0.05% Tween 20); PBST buffer (0.05% Tween 20 in PBS buffer); tris lysed buffer (pH 8.0; 50 mmol/L tris, 150 mmol/L NaCl, 5 mmol/L EDTA with protease inhibitor cocktail); proteinChip washing buffer (0.5% triton X-100 in PBS); rehydration buffer [pH 4–7; 7 M urea, 2 M thiourea, 100 mM DTT, 4% CHAPS, 0.5% carrier ampholytes, 0.01% bromophenol blue (BPB) and 40 mM tris]; equilibration buffer I and II (pH 8.8; 50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, 0.01% BPB, 2 mM DTT/2.5 mM IAA); 10 x MOPS buffer [pH 7.0; 2 M MOPS (morpholinopropanesulphonic acid); 50 mM sodium acetate; 5 mM EDTA]; RNA denaturing buffer (10 ml 100% deionised formamide; 3.5 ml 40% formaldehyde; 1.5 ml 10 x MOPS buffer); TBE buffer (pH 8.3; 0.89 M tris; 0.02 M EDTA; 0.89 M boric acid).

2.1.2.4 Cell culture media and supplements

Standard culture medium contained RPMI 1640 (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal calf serum (Hyclone, Utah, USA). The cell freezing medium was supplemented with 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA).

2.1.2.5 Kits

RC® detergent compatible (DC) protein assay kit, silver stain plus kit, Aurum Serum Protein Mini kit, ReadyPrep 2-D Starter kit, ReadyPrep 2-D Cleanup kit (Bio-Rad Laboratories, Hercules, CA, USA); PHASE RANGE haptoglobin assay kit (Tridelta Development Ltd, Kildare, Ireland); SuperSignal West Pico chemiluminescent substrate kit (Pierce Biotechnology, Rockford, IL, USA); peroxidase ABTS and TMB substrate kit (Vector Laboratories, Burlingame, CA, USA); RNeasy mini kit, one-step RT-PCR kit (Qiagen GmbH, Hilden, Germany); ovarian cancer (CA-125) ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA); human C reactant protein (CRP) ELISA kit (Chemicon International, Temecula, CA, USA).

2.1.3 Hardware

All specialised hardware used in the experiments is listed below with their source.

2.1.3.1 Pipettes, centrifuge tubes, filters

Positive-pressure pipettes (Gilson, Villiers-le-Bel, France); pipette-aid motorised (BD Diagnostics, Franklin Lakes, NJ, USA); 1.5 ml conical-bottom polypropylene tubes, 0.2 µm and 70 µm filters, 70 µm cell strainers and Falcon 25 cm^2 culture flasks (BD Biosciences, Bedford, MA, USA); 1.5 polyallomer tubes (Beckman coulter, Fullerton, CA, USA).

2.1.3.2 Blood collection tubes, needles, slides, coverslips

VACUETTE® serum tubes containing the clot-activating factor (Greiner Bio-One, Kremsmuenster, Austria); needles, uncoated microscope slides, cover slips (BDH Merck, Whitehouse Station, NJ, USA).

2.1.3.3 Centrifuges

Bench top centrifuge for 15- and 50 ml polypropylene tubes (Beckman GS-6R, Beckman coulter, Fullerton, CA, USA); bench top microcentrifuge for 0.5- and 1.5 ml polypropylene tubes (Sanyo Gallenkamp PLC, Loughborough, UK); cytocentrifuge (Shandon, Cheshire, UK); Savant speedvac (Model SC110, Global Medical Instrumentation, Minnesota, USA).

2.1.3.4 Water bath and thermocycler

Standard water bath (GFL, INNOV Solutions, Milan, Italy); ALB64 thermo bath (ESEL TechTra Inc, Korea); Pχ2 programmable thermocycler (Thermo Hybraid, Middlesex, TW, USA).

2.1.3.5 Sonicator

Misonix sonicator (ultrasonic processor)-XL2020 (Misonix incorporated, Farmingdale, NY, USA).

2.1.3.6 Proteomics work station

MP3 electrophoresis system, two dimensional gel electrophoresis systems, ready strips (7cm and 11 cm, pH 3-10 and 4–7), 4-20% tris-HCl precast criterion gel, GS-800 calibrated densitometer, Tran-Blot ® semi-dry transfer cells, electrode wicks, Protean IEF system, UltraRocker rocking platform (Bio-Rad Laboratories, Hercules, CA, USA); nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA); CL-XPosure film (Pierce Biotechnology, Rockford, IL, USA); Ciphergen proteinChip, SELDI-TOF-MS system (Ciphergen Biosystems, Fremont, CA, USA); Voyager-DE STR MALDI-TOF/MS system and ABI 4700 proteomics analyser mass spectrometer with a MALDI source and TOF/TOF™ optics (Applied Biosystems, Framigham, MA, USA), PVC plates (NUNC, Roskilde, Denmark), ELISA reader (Tecan, Salzburg, Austria).

2.1.3.7 Microscope, spectrophotometers and transilluminator

Microscope (Zeiss, Carl Zeiss, Oberkochen, Germany); Beckman DU 650 spectrophotometer (Beckman Coulter, Fullerton, CA, USA); UV transilluminator (UVP Inc, Upland, CA, USA).

2.1.3.8 Computer

IBM R40 (IBM, Armonk, NY, USA).

2.1.3.9 Computer software

Adobe Photoshop graphics software (Adobe Systems UK, Middlesex, UK); Ciphergen software (version 3.0, Ciphergen biosystems, Fremont, CA, USA); Quantity one software, PDQuest version 7.2 software package (Bio-Rad Laboratories, CA, USA); SPSS software (SPSS Inc., Chicago, IL, USA).

2.2 Methods

2.2.1 Sample preparation

2.2.1.1 Cyst fluid sample preparation

The cyst fluid proteins were purified using three different protein precipitation methods namely acetone precipitation, chloroform/methanol precipitation and ammonium sulphate precipitation. For acetone precipitation, four volumes of ice-cold acetone were added into one volume of cyst fluid samples. The mixtures were vortexed and incubated overnight at -20 °C. For the chloroform/methanol precipitation method, five volumes of chloroform/methanol solution (70:30 v/v) mixtures were mixed with one volume of cyst fluid samples. The solution was incubated in ice for 2 hours and the lower organic layer was discarded. For the ammonium sulphate precipitation protocol, ammonium sulphate powder (13.2 mg) was added into 20 µl of cyst fluid and the mixtures were gently vortexed for 10 min. The samples were then incubated in ice for 2 hours. After centrifugation at 10, 000 rpm at 4 °C for 30 min, the supernatant was removed. The pellet was washed using 20 µl of 90% ice-cold acetone and incubated in ice for another 15 min. The samples in all three above mentioned procedures were centrifuged at 13,000 rpm, 4 °C for 30 min. The pellet was air dried for maximum of 5 min and resuspended in millipure water. The purified cyst fluid samples were kept at -80 °C for further analysis.

2.2.1.2 Blood sample preparation

The venous blood samples were collected in 8 ml VACUETTE® serum tubes containing the clot-activating factor (Greiner Bio-One). All samples were transported in ice to the laboratory and centrifuged immediately at 4 °C for 10 min at 1500 g. The supernatant serum aliquots were stored at -80 °C until analysis according to the method described previously (Maccio et al., 2005).

2.2.1.3 Tissue sample preparation

Frozen section slides of tissue were stained using H & E solution and cancer cells with large and irregular nuclei and hyperchromasia were carefully counted under microscope in five randomly selected fields following consultation with the histopathologist wherein the cancer cells should be more than 60% of all cells in the field. Proteins were extracted from tissue using tris lysis buffer for Western blotting procedures.

2.2.2 Cell culture

2.2.2.1 Thawing of frozen cell lines and culture

Three ovarian cancer cell lines were included in this study. TOV-112D, Caov-3 and SKOV-3 cells sent on dry ice or stored in liquid nitrogen were resuscitated by leaving at 37 °C until completely defrosted. The cell suspensions were transferred into 15 ml polypropylene tubes containing 10 ml pre-warmed RPMI culture medium (Invitrogen) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma). After centrifugation at 1000 rpm at room temperature to separate the cells from freezing media containing DMSO

(Sigma), the pellets were resuspended in 10 ml culture media and maintained as monolayers in 75 cm^2 culture flasks (Falcon) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were passaged every 48-72 hours and maintained at an approximate density of 80% confluence.

2.2.2.2 Cryopreservation of cell lines

Immediately before and after cryopreservation (10-14 days) the possible contamination from bacteria, fungi, mycoplasma, and viruses should be checked. If contamination is confirmed, then the frozen material is destroyed. Confluent cells were digested using 0.25% trypsin, collected using gentle centrifugation (10 min at 1000 rpm) and resuspended in the freeze medium (RPMI with 5% DMSO) at a concentration of 1×10^6 to 5×10^6 viable cells/ml. Aliquots of 1.5 ml of solution containing cells were transferred into cryopreservation tubes (Corning). The cells were allowed to equilibrate in the medium at room temperature for a minimum of 15 min and the tubes were transferred into a controlled-rate freeze chamber and placed in a –70 °C mechanical freezer for at least 24 hours. The cells were deposited into the liquid nitrogen bank the following day.

2.2.3 Protein quantification

The cyst fluid protein concentration of each sample was determined using the Bradford method (Bio-Rad Laboratory). For the standards, 10 µl to 80 µl of BSA solution of 1 µg/µl was added to 390 µl, 380 µl, 370 µl, et al. of autoclaved water respectively. Samples were diluted 1:10 before quantification. Ten µl of sample was added to 390 µl of autoclaved water. Both the standards and the samples were mixed with 100 µl of Bradford dye (Bio-Rad Laboratory) according to the manufacturer's instructions. The total volume in each cuvette is 500 µl. The

samples were then analysed at 595 nm using a spectrophotometer (Beckman Coulter).

Protein concentrations of tissue proteins were determined using Bio-Rad RC® DC protein assay kit (Principle of Lowry method) following the manufacturer's instructions with slight modifications. Twenty-five µl of BSA standards (0.2 µg/µl, 0.6 µg/µl, 1 µg/µl and 1.4 µg/µl) or samples were mixed with RC® DC reagent I and II, and the mixtures were centrifuged at 13,000 rpm for 10 min. The supernatants were discarded and the pellets were dissolved in 127 µl of working reagent A (20 µl of reagent S to each ml reagent A). After incubation with 1 ml of reagent B for 15 min, the absorbance of the solution was read at 750 nm. Protein concentrations were calculated using the equation deduced from the standard curve.

2.2.4 SELDI-TOF-MS Analysis

SELDI-TOF profiling for hydrophilic cyst fluid proteins was obtained using normal phase (NP20-hydrophilic surface) proteinChips (Ciphergen Biosystems). The proteinChips were pre-incubated with 5 µl of Millipure water for 5 min. 5 µl of sample was then added to the NP20 proteinChip spots and incubated for 1 hour. Immunocapture experiments were performed using a PS20 ProteinChip array (Ciphergen Biosystems) precoated with anti-haptoglobin antibody (Dakocytomation). Samples (10 µl) were incubated with 90 µl binding buffer (0.5% Triton X-100 in PBS) and allowed to bind the chips for 2 hours. After two washes with binding buffer, the above mentioned arrays were treated with saturated sinapinic acid in 0.5% trifluoroacetic acid and 50% acetonitrile and analysed using the Ciphergen proteinChip Reader (model PBSII; Ciphergen Biosystems). The mass spectra of proteins were generated using an average of

80 laser shots at each sample. For low molecular weight proteins, data collection was optimised between 8-25 kDa. The detector intensity was set at 10, and the laser intensity was set at 240 V. For middle size proteins, data collection was optimised between 25-50 kDa. The detector sensitivity and laser intensity was 10 and 260 V, respectively. The mass to charge ratio (m/z) of each of the proteins captured on the array surface was determined according to externally calibrated standards (Ciphergen Biosystems): bovine insulin (5,733.6 Da), human ubiquitin (8,564.8 Da), bovine cytochrome c (12,230.9 Da), equine myoglobin (16,951.5 Da), bovine β-lactoglobulin A (18,363.3 Da) and horse-radish peroxidase (43,240 Da). Mass spectra were processed using the Ciphergen proteinChip software version 3.0 for baseline correction and peak detection in the auto mode. Baseline subtraction was performed on spectra to eliminate any baseline signal that was due mainly to chemical noise caused by EAM. Peak was identified as areas of the mass spectrum by comparing the signal to neighbouring valley depths with a signal to noise ratio of greater than 5.

2.2.5 SDS-PAGE

2.2.5.1 Assembly of apparatus before casting the polyacrylamide gels

For preparation of casting the polyacrylamide gel, the 0.75 mm spacer and short plates from Bio-Rad Laboratories are cleaned with 70% ethanol. The cleaned 0.75 mm spacer and short plates are inserted into the casting frame, placed on the gasket and held together by the casting stand.

2.2.5.2 Preparation of SDS-PAGE

SDS-PAGE gels with different acrylamide concentrations ranging from 8 to 15% were used in the present study. Compositions for the mini-size (7 cm) gels were described in Table 2.1. Resolving gel mixture was prepared by mixing the reagents listed in Table 2.1 and loaded into the space between the glass plates. Next, stacking solution was loaded above the polymerised resolving gel by adding slowly at the side to the brim of the short plate. The comb was then Inserted (Bio-Rad Laboratories) and the acrylamide in the stacking solution was allowed to polymerise for about 15 min. The entire assembly was left overnight at 4 °C before usage.

2.2.5.3 Running SDS-PAGE

Protein samples were premixed with loading buffer and heated at 95 °C for 5 min. Once acrylamide was polymerised, protein standards and samples were loaded and electrophoresis was performed by applying a voltage of 120 V. Enhanced resolution of low molecular weight protein bands was obtained by preparing high (12%-15%) percentage gel and vice versa.

Table 2.1 Composition of mini size SDS-PAGE gel

Components of single gel	Stacking gel mixture	Separating gel mixture			
		8%	10%	12%	15%
H_2O	1.75 ml	4.35 ml	3.75 ml	3.15 ml	2.25 ml
4xTris(Upper/Lower)	625 µl	2.25 ml	2.25 ml	2.25 ml	2.25 ml
30%AA	325 µl	2.4 ml	3 ml	3.6 ml	4.5 ml
10%APS	30 µl	25 µl	25 µl	25 µl	25 µl
TEMED	12 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl

4x Lower tris (pH 8.8; 1.6 M tris, 0.4% SDS)

4x Upper tris (pH 6.8; 0.5 M tris, 0.4% SDS)

AA: acrylamide/bis solution

APS: ammonium persulphate

2.2.6 Native gel electrophoresis

Native gel electrophoresis runs in the absence of SDS and the mobility of proteins depends on both the charge and hydrodynamic size of the proteins (Krause, 2006). Native gels with different acrylamide concentrations ranging from 10 to 15% were used in the present study. Compositions for the mini-size (7 cm) gels are described in Table 2.2. Protein samples were premixed with loading buffer (pH 6.8; 0.313M Tris-HCl, 0.05% bromophenol blue, 50% glycerol). Once acrylamide is polymerised, protein standards and samples were loaded and electrophoresis was performed by applying a voltage of 120 V.

Table 2.2 Composition of mini size native gel

Components of single gel	Stacking gel mixture	Separating gel mixture		
		10%	12%	15%
H_2O	2.5 ml	5 ml	3.7 ml	2.5 ml
Tris (Upper/Lower)	1 ml	2.8 ml	2.8 ml	2.8 ml
30%AA	0.4 ml	3.7 ml	4.5 ml	5.7 ml
10% APS	40 µl	40 µl	40 µl	40 µl
TEMED	10 µl	10 µl	10 µl	10 µl

Lower tris (PH 8.8; 1.5M tris-HCl)
Upper tris (PH 6.8; 0.5M tris-HCl)
AA: acrylamide/ bis solution
APS: ammonium persulphate

2.2.7 Silver staining

The protein bands were visualised using silver stain plus kits (Bio-Rad laboratories) according to manufacturer's instructions with modifications. After electrophoresis, gels were taken out from the glass plates and placed with care into appropriate plastic containers. The gels were then fixed in approximately 25 ml of fixative solution containing 40% methanol and 10% acetic acid (v/v) for at least 30 min on an UltraRocker Rocking Platform (Bio-Rad Laboratories) at room temperature. After washing by deionised distilled water for three times, gels were stained in developer solution until desired staining intensity was reached. Next, the gels were placed in 5% acetic acid to stop the reaction (minimum 15 min) and protein bands were scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories).

2.2.8 2-DE

2.2.8.1 Sample preparation

2.2.8.1.1 Removal of high abundance protein

The high abundance protein albumin was removed using Aurum Serum Protein Mini kits (Bio-Rad Laboratory). The protein column was placed in a 12 x 75 mm test tube and the resin was allowed to settle for at least 5 min. Gravity flow started after removal of caps and tips from the bottom of the columns and the residual buffer was allowed to drain from the column (approximately 2 min). Next, the column was washed using 1 ml of protein binding buffer twice using gravity flow. After centrifuging the column at 10,000 rpm for 20 sec in a microcentrifuge to dry the resin bed, 200 µl of the diluted cyst fluid sample in binding buffer (1:3, v/v) was added to the top of the resin bed and the sample was allowed to penetrate the column matrix. This was followed by gentle vortexing for three times at 5 min intervals and the column was centrifuged at 10,000 rpm for 20 sec. The eluate in the collection tube was collected which contains the albumin depleted cyst fluid samples.

2.2.8.1.2 Sample cleanup

Protein samples were purified using ReadyPrep 2-D Cleanup Kit. Proteins were mixed with three volumes of precipitating agent 1 in a 1.5 ml microcentrifuge tube and the tube was allowed to incubate on ice for 15 min. The same volume of precipitating agent 2 was added to the mixture and the tubes were centrifuged at 13,000 rpm for 5 min. Next, the supernatant was discarded without agitating the pellet followed by brief centrifugation again to remove any residual liquid. The

pellets were then washed using wash reagent 1, proteomic grade water and wash reagent 2 sequentially. After centrifugation, the pellets were resuspended by adding an appropriate volume of 2-D rehydration/sample buffer.

2.2.8.2 First-dimension separation

In total, 100 µg of cyst fluid protein was mixed with rehydration buffer to a final volume of 200 µl and the mixtures were vortexed 4 times for 20 sec at 1 min intervals. This mixture was then applied to Ready Strips (11 cm, pH 3-10, 4–7, Bio-Rad Laboratories) and the rehydration was conducted at room temperature for 16 hours. Next, cyst fluid proteins were isoelectrically focused at 50 V for 2 hours; 250 V for 1 hour; 500 V for 1 hour; 1000 V for 1 hour; 8000 V for 3 hours and then maintained at 8000 V for a total of 60000 Vhs. Ready Strips were then stored at -80 °C until second dimensional electrophoresis.

2.2.8.3 Second-dimension separation

Ready Strips from the first dimensional separation were equilibrated in 3 ml of equilibration buffer I and buffer II for 15 min each. Strips were rinsed in tris glycine SDS running buffer and then applied to the top of a 4-20% precast criterion gel (Bio-Rad Laboratories). Low melting point agarose (0.5% in running buffer containing BPB) was layered on top of the strip and electrophoresis on the gel was carried out at 150 V for 1 hour.

2.2.8.4 Gel staining and image analysis

The protein spots were visualised using silver stain plus kits (Bio-Rad laboratories) Protein spots were scanned using a GS-800 calibrated densitometer

(Bio-Rad Laboratories) followed by spot detection, quantification, and matching with the PDQuest version 7.2 software package (Bio-Rad Laboratories). The gel spots were automatically detected by the computer program and the expression levels of proteins were determined by averaging the normalisation volume of the spots. The differentially expressed proteins between diseases and controls were selected and identified.

2.2.9 MALDI TOF/MS and MALDI TOF-TOF/MS

2.2.9.1 In-gel digestion for mass spectrometry

In-gel digestion was performed as follows. Finely cut gel pieces were ground and transferred into a 600 µl PCR tube. Destaining was performed by the addition of 1:1 ratio of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate solution followed by vortexing. The mixture was then washed and dehydrated sequentially in 100 mM ammonium bicarbonate, 50 mM ammonium bicarbonate and 50% acetonitrile (v/v). After the sample was dried in a vacuum centrifuge it was reduced by adding a fresh solution of 10 mM DTT in 100 mM ammonium bicarbonate (incubated at 57 °C for 60 min), and alkylated by using 55 mM iodoacetamide solution in 100 mM ammonium bicarbonate (incubated at room temperature for 60 min). Further procedures included gel washing in 100 mM ammonium bicarbonate (twice), dehydration in 100% acetonitrile (twice), re-swelling in 100 mM ammonium bicarbonate (once), dehydration in 100% acetonitrile (twice), and drying in a vacuum centrifuge (once). The dried sample was reconstituted by adding digestion solution (12.5 ng/µl trypsin in 50 mM ammonium bicarbonate solution), and kept at 4 °C for 30 min. The excess digestion solution was removed and 50 mM ammonium bicarbonate solution was added to the sample before incubation in a thermo bath at 37 °C (12-15 hours).

After incubation, the solution was centrifuged at 6,000 rpm (10 min) and supernatant ("fraction A") collected; to the pellet, 20 mM ammonium bicarbonate was added and spun at 6,000 rpm (10 min) and the supernatant ("fraction B") was collected; to the pellet, 5% formic acid in 50% aqueous acetonitrile was added and centrifuged at 6,000 rpm (10 min) and supernatant ("fraction C") was obtained. The three fractions ("A", "B" and "C") were pooled and dried in a vacuum centrifuge.

2.2.9.2 MALDI TOF analysis

After in-gel digestion, the vacuum dried sample containing peptides was dissolved in 0.1% TFA and diluted in 50% acetonitrile. 0.4 µl of the extracted sample was dispensed onto a MALDI sample plate along with 0.4 µl of matrix solution consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA), 0.1% TFA, and 50% acetonitrile. Mass spectrometric analysis was performed using Applied Biosystems Voyager-DE STR MALDI-TOF mass spectrometer with the following specifications. MS automatic data acquisition was performed in delayed extraction, reflectron mode, with the parameters set as follows: accelerating voltage of 20,500 V; grid voltage of 73.5%; guide wire voltage of 0.01% and extraction time of 380 ns. Laser intensity was set at 2700 with 100 shots per spectrum. The mass range was set between 800-3500 Da. The mixture of ACTH peptides (1296.6835 Da and 2465.1989 Da ($M+H^+$) ions) were used as internal calibration standards.

2.2.9.3 MALDI TOF-TOF analysis

Extracted peptides were resuspended in solution with 0.1% (v/v) TFA and 50% (v/v) acetonitrile. 0.5 µl of this peptide solution was mixed with an equal volume

of matrix solution containing saturated solution of CHCA in 0.1% (v/v) TFA and 50% (v/v) acetonitrile and spotted onto a MALDI target plate. The sample was analysed on the MALDI TOF/TOF 4700 Proteomics Analyser (Applied Biosystems). Calibration of the instrument was performed externally with $[M+H]^+$ ions of calibration mixture (4700 Cal Mix). For MS analysis, the instrument was turned in a reflector mode with the laser intensity set at 5% higher than the threshold for ionisation. MS spectra were obtained after accumulation of 5000 consecutive laser shots. For MS/MS analysis, the laser intensity was set at 15% higher than the threshold for ionisation. Nitrogen was used as collision gas and a medium pressure was selected (approximately $8x10^{-7}$ torr as measured by the source 2 pressure gauge). Data-dependent MS/MS analysis was performed in automatic mode with the highest twenty MS peaks in intensity selected for MS/MS analysis.

2.2.9.4 Database search and bioinformatics analysis

The search engine MASCOT was used for both peptide mass fingerprinting (PMF) and MS/MS based sequence query. For MASCOT PMF search, parameters were set as follows: database was chosen with NCBI nonredundant database, taxonomy was selected as Homo sapiens, protein mass was set at 0-250 kDa, and one missing cleavage was allowed. Methionine oxidation and cysteine carbamidomethylation were selected as variable modifications. Peptide masses were stated to be monoisotopic and the mass tolerance was set as 50 ppm.

For MASCOT sequence query search the parameters were set as follows: PMF data in combination with MS/MS spectra were submitted to the MASCOT database sequence query search engine using mass tolerance settings of 100 ppm for PMF and 0.2 Da for MS/MS data, and 1 missing cleavage site. The

protein mass was set at 0-250 kDa. Taxonomy was chosen as Homo sapiens. Peptide masses were stated to be monoisotopic. Methionine oxidation and cysteine carbamidomethylation were selected as variable modifications. Trypsin autodigested peaks and keratin trypsin digested peaks were excluded from the submitted peaks.

2.2.10 Western blotting analysis

To perform Western blotting analysis, proteins were separated using SDS-PAGE as described in section 2.2.5. The separated proteins in the acrylamide gel were transferred onto a nitrocellulose membrane using Tran-Blot ® semi-dry transfer cell (Bio-Rad Laboratories) at 15 V for an hour. Next, the membrane was incubated with a blocking solution consisting of 5% non-fat dry milk in TBST solution at 37 °C for 1 hour followed by incubation with primary antibody overnight at 4 °C. After washing the membrane 3 times (10 min each) with TBST, the membrane was incubated with a secondary antibody conjugated with HRP at a 1:5000 dilution for 1 hour at room temperature. The membrane was briefly incubated with chemiluminescence HRP substrate (250 µl of peroxide solution and 250 µl of enhancer solution; Pierce) and exposed in the dark room to X-ray film for the appropriate time period (about 10 sec, 1 min and 3 min) to visualise the chemiluminescence signal corresponding to the specific antibody-antigen reaction. Band intensities were measured using Quantity One® software (Bio-Rad Laboratories) in order to obtain any quantitative difference of protein expression between the benign and malignant ovarian tumours.

2.2.11 Antibody generation

Polyclonal rabbit anti-human antibody was generated from Biogenes GmbH Pte Ltd (Biogenes). Specific antigenic peptides were obtained by analyses of complete or partial protein sequences based upon the antigenicity, hydrophilicity, and accessibility parameters, which increases the likelihood of generating an antibody that recognises the full length protein. A sequence representing haptoglobin α_2-subunit was produced (CKNYYKLRTQGDGVY) based on these criteria and the specificity of this recommended antigenic peptide was confirmed using the Basic Local Alignment Search Tool (BLAST). This procedure is very important since short peptides may have higher degree of amino acid homology with other proteins therefore creating unwanted antibody cross reactivity. After generation of the specific peptide, the laboratory rabbits were inoculated with this peptide. A first boost was made one week later and the trial bleeds were recovered from rabbits 10 days after each boost injection. The titre evaluation of the antiserum was performed using an indirect immunoassay method. Once the protocol of immunisation was found satisfactory to elicit the targeted antibodies, larger amounts of antiserum were produced using several immunisations in rabbits. Antisera with similar characteristics (affinity, titre and crossreactivity) were pooled to allow a consistent source of antibodies.

2.2.12 ELISA

Polyclonal rabbit anti-human haptoglobin α-subunit antibody was generated using amino acid sequence, CKNYYKLRTQGDGVY (BioGenes). The affinity-purified antibody developed for the haptoglobin α-subunit and two commercially available antibodies for the whole haptoglobin molecule were used for the sandwich ELISA analysis. The total haptoglobin and haptoglobin α-subunit levels were quantified

using sandwich ELISA with rabbit anti-human polyclonal antibodies against haptoglobin (Dakocytomation) or haptoglobin-α subunit. Goat anti-human haptoglobin antibody (Biodesign) diluted in 0.5% BSA in PBST buffer (1:200) was coated onto 96-well plates (NUNC) and the plate was incubated overnight at 4 °C. The plates were then blocked with 200 µl 3% non-fat dry milk in PBST for 2 hour at room temperature. Next, diluted cyst fluid samples were individually mixed with 0.5% BSA in PBST buffer (1:100) and the mixture was incubated for 2 hour at room temperature. The purified human haptoglobin (Sigma-Aldrich) or haptoglobin α-subunit (BioGenes) was used as standards. The plate was then incubated with rabbit anti-human haptoglobin (1:400) (Dakocytomation) or haptoglobin-α subunit antibody (1:100) diluted in 0.5% BSA in PBST at room temperature for 2 hours followed by biotin-labelled anti-rabbit secondary antibody (1:400) and alkaline phosphatase streptavidin (1:400; Vector Laboratories). p-Nitrophenylphosphate (Vector Laboratories) was applied at 5 mM in 100 mM sodium bicarbonate solution at 37 °C for about 1 hour. The protein concentrations were determined at 405 nm using an ELISA reader (Tecan).

2.2.13 Measurement of haptoglobin using the PHASE RANGE haptoglobin assay kits

A commercially available PHASE RANGE haptoglobin assay kit (Tridelta Development Ltd) was sourced that allowed a rapid, semi-quantitative assessment of total haptoglobin concentrations within body fluids. This assay is based on the fact that the peroxidase activity of free haemoglobin is inhibited at a low pH environment. Binding of haptoglobin with haemoglobin will preserve the peroxidase activity of the bound haemoglobin. Hence the peroxidase activity is proportional to the amount of haptoglobin present in the specimen. We determined the accuracy and clinical usefulness of this kit in 90 cyst fluid samples.

Endometriotic and dermoid cysts were excluded from the study. 47 benign epithelial ovarian cysts, 13 borderline, 10 early stage and 20 late stage ovarian cancers were subjected to this analysis. The assay was performed in a 96-well plate following the manufacturer's instructions. 7.5 µl each of either prepared haptoglobin calibrators (0-2mg/ml), or the test specimens, were loaded in duplicate into the wells. This was followed by the 100 µl of haemoglobin solution and mixed. 140 µl of a mixture of chromogen and substrate were next added and the plate was incubated for 5 min. The colour reaction was recorded at the end of 5 min.

2.2.14 Evaluation of ovarian tumour using ultrasonographic scoring system

Transvaginal ultrasound examination was performed on an empty urinary bladder using a sector transducer (5-8 MHz) with a 210^{o} /240^{o} image section (Acuson Sequoia Echo Ultrasound System, CA, USA). Ultrasonographic evidence of metastasis and ascites are reported only if obvious, but we routinely report (1) the presence or absence of an ovarian tumour, (2) its solid or cystic nature, (3) the presence of septae and loculations within the tumour, (4) the presence of solid areas or papillary projections within the cyst, (5) whether the tumour is unilateral or bilateral, and (6) vascularity.

2.2.15 Measurement of CA-125 level in serum and cyst fluid using sandwich ELISA method

Serum and cyst fluid CA-125 levels were determined using a CA-125 ELISA kit (Alpha Diagnostic International). The samples were diluted ten times using wash buffer and 25 µl of standards, control, and samples were pipetted into a 96-well plate in duplicate. Next, 100 µl of biotinylated anti-CA-125 capture antibody was

loaded and the plate was incubated for 2 hours at room temperature. After washing, 100 µl of HRP conjugated anti-CA-125 antibody was added and the plate was incubated for 1 hour at room temperature followed by addition of substrate and the stop solution. The absorbance was read at 450 nm using the ELISA reader (Tecan).

2.2.16 Histochemical and immunohistochemical studies

The ovarian tumour tissues were frozen in liquid-nitrogen-cooled isopentane for histochemical examination. Sections of 8 µm were cut from frozen slices and haematoxylin and eosin staining was performed to visualise sections containing the malignant ovarian cancer cells. Briefly, in a slide rack, they were immersed in haematoxylin for 15 min, rinsed in running water, dipped in acid ethanol for 10 sec and washed in running tap water for 5 min. After 15 min in eosin, the slides were dehydrated in an ethanol series (70%, 100% x2), air-dried and mounted with coverslips over DPX mountant. For immunohistochemistry study, after 8 min blockage with ultra v block reagent (BioGen Medical Instruments Inc), sections from ovarian cancer and benign ovarian tissue were immunostained for 1 hour using rabbit anti-human ceruloplasmin and haptoglobin antibody (Dakocytomation) at 37 °C followed by incubation with biotinylated anti-rabbit secondary antibody (1 hour at room temperature) and streptavidin alkaline phosphatase (30 min at room temperature) according to the manufacturer's instructions. Sections from formalin-fixed, paraffin-embedded tissues were also included in this study. The slides were deparaffinised, treated with 3% hydrogen peroxide in phosphate-buffered saline and pretreated at 95 °C for 10 min in 10 mol/l citrate buffer (pH 6.0). Diaminobenzidine was used as the chromogen and haematoxylin as the nuclear counterstain. IgG was used as negative control to replace the primary anti-haptoglobin antibody. Tumours were considered positive

for ceruloplasmin and haptoglobin if definite cytoplasmic staining was present when observed under the microscope.

2.2.17 Reverse transcription polymerase chain reaction (RT-PCR)

2.2.17.1 Total RNA extraction

Total RNA was isolated from cancer cells using an RNeasy Mini Kit (Qiagen) with minor modifications according to the manufacturer's instructions. Briefly, cancer cells ($1x10^6$) were mixed with 350 µl of Buffer RLT in 2 ml tubes. After vortexing and homogenising the mixture, 350 µl of 70% ethanol was added to the homogenised lysates. Next, 700 µl of this lysates containing any precipitate formed were applied to an RNeasy mini column placed in a 2 ml collection tube followed by centrifugation at 10,000 rpm for 15 sec. After washing with 350 µl Buffer RW1, the samples were incubated with DNase for 30 min at room temperature. The column was then washed using 700 µl Buffer RW1 and 500 µl Buffer RPE sequentially. Thirty to fifty micro litre of Rnase-free water was added directly onto the Rneasy silica-gel membrane to dissolve the RNA. After centrifugation, the eluate was collected and the quantity of RNA was estimated.

2.2.17.2 Quantitative analysis of RNA products

The concentration of RNA isolated was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a spectrophotometer (Beckman). The ratio of absorbance at A_{260}/A_{280} was taken as a measure of purity of total RNA. 2 µl of RNA was mixed with 78 µl of TE buffer (pH 7.0; 10 mM Tris, 1 mM EDTA). After reading A_{260} blanked against TE buffer, the RNA recovered was determined by the following fomula: Total RNA (µg/ml) = (A_{260}) x 40 µg /ml/ A_{260} x 40.

2.2.17.3 Qualitative analysis of RNA products

The integrity and size distribution of total RNA purified was determined by denaturing agarose gel electrophoresis and ethidium bromide staining. A 1.2% agarose gel was prepared by suspending appropriate amounts of agarose in DEPC treated water. To the melted and cooled agarose, 1x MOPS buffer followed by 3 ml of 37% formaldehyde was added into 100 ml of gel solution. Next, the mixture was swirled and poured into the gel-casting unit and allowed to set. The gel was covered with saran wrap in order to avoid evaporation of formaldehyde followed by immersion in 1x MOPS running buffer in the agarose gel electrophoresis apparatus.

Two µl of the 10x loading buffer (2 µl 5x MOPS buffer, 3.5 µl formaldehyde and 10 µl formamide) was added into 20 µl of RNA samples. The mixture was denatured by heating at 65 °C for 15 min and applied to agarose gel electrophoresis at 50 V. When electrophoresis was completed, the gel was immersed into distilled water with 0.2 µg/ml ethidium bromide at room temperature for 5 min, viewed in a UV transilluminator (UVP Inc) and photographed.

2.2.17.4 RT-PCR

Gene transcripts levels were determined using RT-PCR method. A total reaction volume of 50 µl (Table 2.3) containing Sensiscript reverse transcriptase and HotStarTaq DNA polymerase, 400 µM dNTP, 1X reaction buffer, 0.6 µM of genetic specific primers, and 1 µg of template RNA were amplified in a Pχ2 programmable thermal cycler (Thermo Hybraid) (Table 2.4). To ensure that only RNA was amplified, a negative control in which the RT enzyme was omitted was

performed for each sample. After amplification, the PCR products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 g/ml) and visualised under UV light.

Table 2.3 Components for one-step RT-PCR reaction

Component	Volume/reaction µl	Final concentration
RNase-free water	34.4	
5x Qiagen OneStep RT-PCR buffer	10	1x
dNTP Mix	2.0	400 µM
Haptoglobin Primer forward	0.3	0.6 µM
Haptoglobin Primer B reverse	0.3	0.6 µM
Enzyme Mix	2.0	
Template RNA		1 µg
Total	50	

Table 2.4 Condition for one-step RT-PCR reaction

	Time (min)	Temperature (°C)
Reverse transcription:	30	50
Initial PCR activation step:	15	95
3-step cycling (40 cycles)		
Denaturation:	0.5-1	94
Annealing:	0.5-1	52
Extension:	1	72
Final extension:	10	72

2.2.18 Statistical analysis

Statistical significance was evaluated using the Student's *t*-test or ANOVA analysis for comparison of the concentrations among benign, borderline tumour, early and late stage ovarian cancers. For survival analysis, the Pearson's χ^2 test was used to compare the relationship between serum haptoglobin level with demographic and clinical data. The univariate Kaplan-Meier analysis was used

to estimate the probability of survival and differences in survival rates were assessed by the log-rank test. Multivariate analysis of prognostic factors was estimated using the Cox regression model. Correlation studies were conducted using the Pearson's correlation analysis to evaluate any relationship in the levels of proteins of interest in this study. Receiver operating characteristic (ROC) curves were performed to assess the diagnostic performance of biomarkers in cyst fluid using split-point analysis based on previous study (Zhang et al., 2004). Thresholds for each biomarker were set to minimise the false positive and false negative ratios. All statistical analyses were performed using SPSS 13.0 package (SPSS Inc). Results were considered statistically significant at $P < 0.05$.

Chapter 3: SELDI-TOF based identification of differentially expressed cyst fluid protein biomarkers for EOC

3.1 Introduction

Proteins derived from ovarian tumour cells can be detected in the cyst fluid (Hazelton et al., 1999; Furuya et al., 2000; Ott et al., 2003). It is possible that rapid cellular turnover during malignant transformation could contribute to a large array of proteins which may be deregulated in the carcinogenic process. Such tumour-derived proteins could play an important role in the growth of ovarian epithelial neoplasms. By studying proteins secreted by these tumour cells into the cyst fluid, potential tumour markers that are being differentially expressed could be of use to discriminate benign cysts from EOC.

Proteomics is a high-throughput technology which has the capability to identify hundreds of protein molecules present in the patient's serum, biofluids and tissues. The proteinChip platform technology SELDI-TOF, is a recent technology that has been invaluable in the discovery of new biomarkers and also in establishing differential protein profiles in various pathological states including cancers (Cheung et al., 2004; Yang et al., 2005; Engwegen et al., 2007). In this regard, serum proteomic patterns have been utilised with a very high diagnostic power in ovarian cancer (Petricoin et al, 2002). In addition, SELDI-TOF based identification of protein markers has been reported to be the major technique in biomarker detection for EOC (Ye et al., 2003; Moshkovskii et al., 2005).

In this study, a comprehensive investigation using SELDI-TOF, SDS-PAGE, MALDI-TOF alongwith the traditional immunological validation methods was conducted with a view to characterise novel proteins that may be differentially expressed in such tumours. The hypothesis1 of the thesis "Differentially expressed proteins may be present in cyst fluid from benign and malignant ovarian tumours and could be identified using a combination of SELDI-TOF based proteomics methods and traditional immunological techniques" was examined in this chapter.

3.2 Protein profiling using SELDI-TOF technology

Aim. To identify differentially expressed proteins in cyst fluid from benign and malignant ovarian tumours.

Investigation. Differences in protein profiles using the SELDI-TOF technique are represented as unique peaks or peaks with differential intensity within the Ciphergen spectra in diseases when compared with controls. Detection of these protein peaks could be carried out either manually or with the help of Ciphergen ProteinChip software version 3.0.1 (Ciphergen Biosystems Inc). The signature protein profiles of interest could be further fractionated using gel-based methods and identified by MALDI TOF-TOF/MS. Sample preparation for hydrophilic NP20 ProteinChip arrays as well as optimised machine settings for data acquisition from SELDI-TOF was described in section 2.2.4.

Results and Interpretation. The discriminating pattern formed by a small subset of proteins or peptides among the entire repertoire of thousands of proteins present in cyst fluid samples could distinguish malignant from benign ovarian

tumours. Using Ciphergen's Biomarker Program, it revealed that the protein profiles in benign and malignant cyst fluid samples shared many similarities among their hydrophilic proteins. Figure 3.1 A illustrates the representative SELDI-TOF profiles ranging from 5 kDa—20 kDa according to the m/z ratio. A major difference was observed from the 17.5 kDa peak in cyst fluid obtained from late stage ovarian cancers. This particular peak was greatly diminished or absent in the benign tumours of the ovary (Figure 3.1 A; 3.1 B and 3.1C). The normalised 17.5 kDa protein peak intensities in cyst fluid from benign, borderline tumour, early and late stage ovarian cancer is given in Table 3-1. It was observed that this peak of high intensity was present in 9 out of 10 late stage ovarian cancers. In contrast, it was not detected or was present with very low intensities in the majority of benign ovarian tumours. Moreover, statistical analysis using ANOVA showed that the peak intensities for this 17.5 kDa protein were significantly higher in late stage ovarian cancer when compared with those from benign ovarian tumours ($P<0.01$). Hence, the 17.5 kDa protein possessed the ability to differentiate malignant from benign ovarian tumours. However, the identity of this protein could not be ascertained using the SELDI-TOF technique. In this regard, we utilised the conventional SDS-PAGE method to separate this protein based on the molecular weight and identify it using MALDI TOF-TOF/MS method.

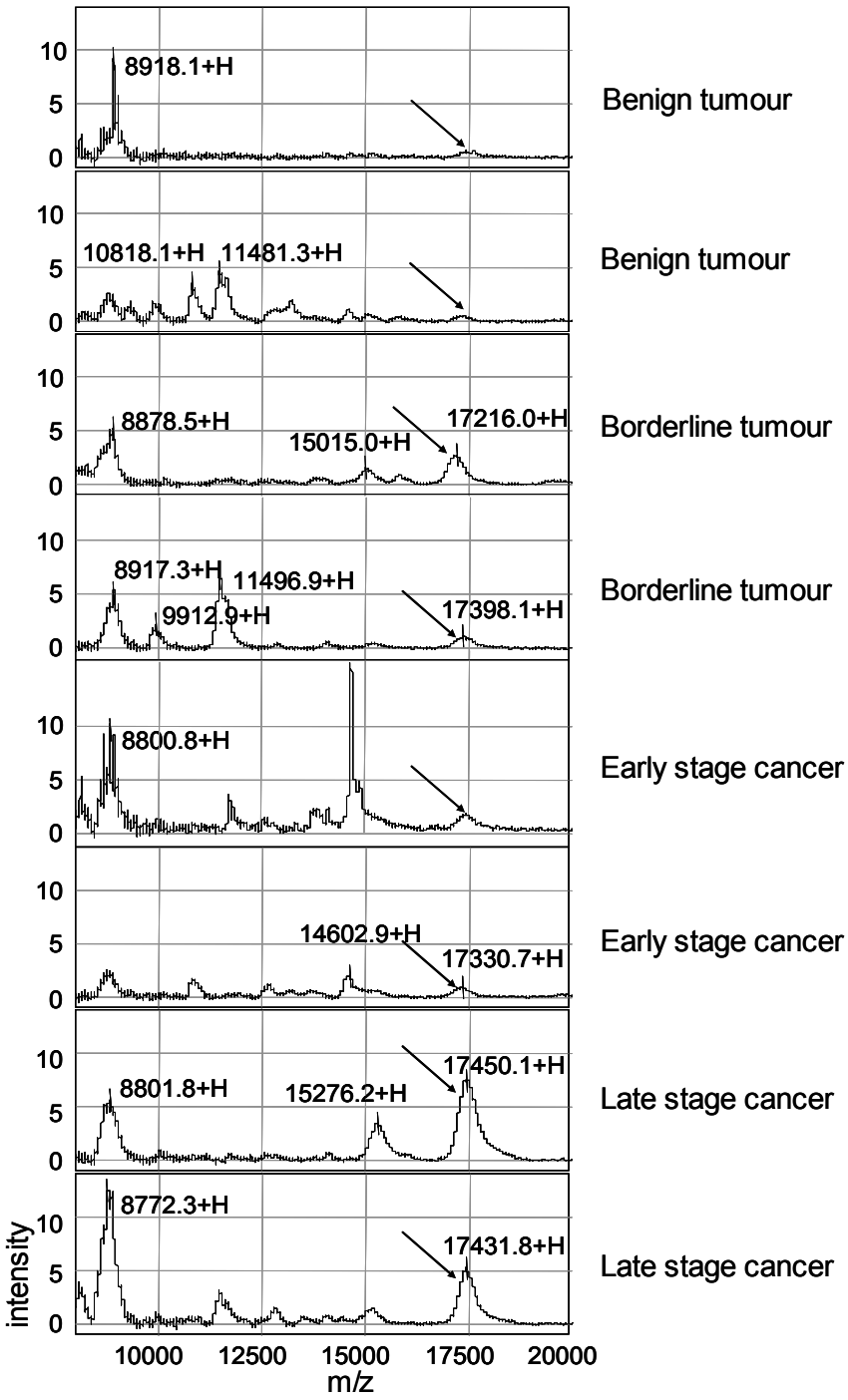


Figure 3.1 SELDI-TOF protein profile analysis

A Cyst fluid protein profiles are demonstrated between 5-20kDa according to m/z ratio using NP20 protein chips. Arrows indicate the position of the 17.5 kDa peak observed in ovarian carcinomas which was less frequently observed in benign tumours.

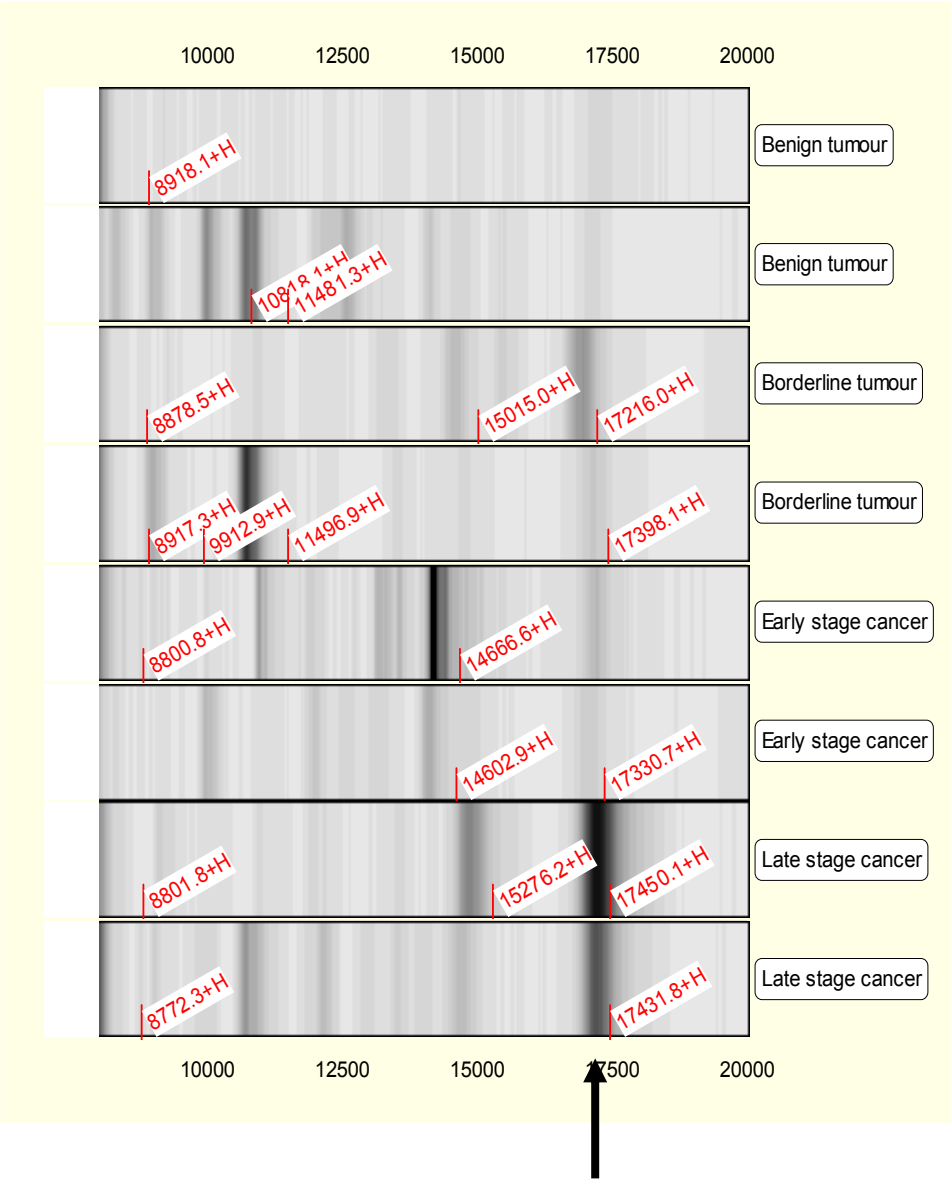


Figure 3.1 B The identified protein peak at 17.5 kDa is indicated in the gel view (arrow) from SELDI-TOF protein profiles

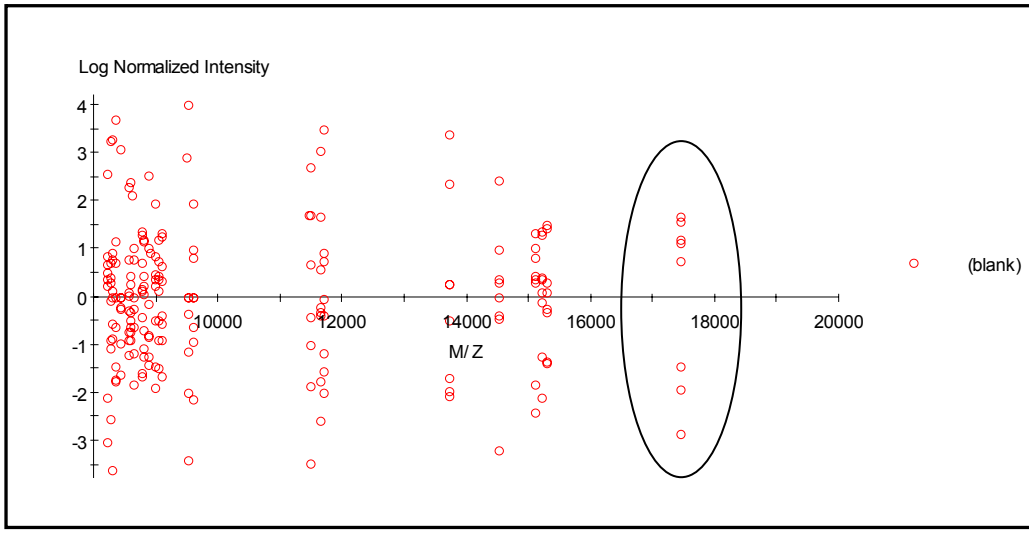


Figure 3.1 C The 17.5 kDa protein peak (circled) which has the ability to distinguish the malignant from the benign ovarian tumours is shown using Ciphergen Biomarker Wizard software

Table 3.1 Normalised 17.5 kDa protein peak intensities in cyst fluid from benign and malignant ovarian tumours

Benign		Early or borderline		Late	
Sample No.	Intensity	Sample No.	Intensity	Sample No.	Intensity
1	0.9	10	2.4	11	2.5
2	ND	20	1.6	13	ND
3	0.6	26	1.6	14	7.3
4	0.9	27	0.9	15	5.2
5	3.9	74	1.4	16	2.9
6	2.7	25	1.1	17	4.6
7	0.7	31	1.7	18	2.1
8	ND			32	2.7
9	1.0			46	6.8
21	2.9			49	4.3

ND: peak not detected

3.3 Protein fractionation and identification using SDS-PAGE and MALDI TOF-TOF/MS

3.3.1 Optimisation of protein precipitation methods for SDS-PAGE

Aim. Protein samples commonly contain substances that interfere with downstream applications. There are several strategies for removing these substances, of which protein precipitation is one of the easiest methods utilised in the purification and concentration of proteins. Protein precipitation is generally induced by addition of a salt, an organic solvent or by altering the pH of the solution (Rothstein, 1994). Several precipitation methods were tested in order to precipitate proteins optimally from cyst fluid in this study.

Investigation. Cyst fluid samples were collected from patients presenting with benign and malignant ovarian tumours after obtaining informed consent as approved by the ethics committee of the National University Hospital (section 2.1.1.1). Proteins were precipitated using three different methods including i) acetone, ii) chloroform/ methanol and iii) ammonium sulphate precipitation (section 2.2.1.1). After quantification using Bradford assay (section 2.2.3), ten micrograms of protein was separated by 12% SDS-PAGE followed by visualisation using silver stain (section 2.2.5). Band intensity was detected visually or with the aid of Quantity One® software (Bio-Rad Laboratories).

Results and interpretation. Cyst fluid proteins separated by SDS-PAGE and observed by silver stain method is given in Figure 3.2. It was observed that the proteins extracted using acetone had a better band recovery when compared with the other two precipitation methods. Therefore, this method of precipitation was used in all subsequent experiments.

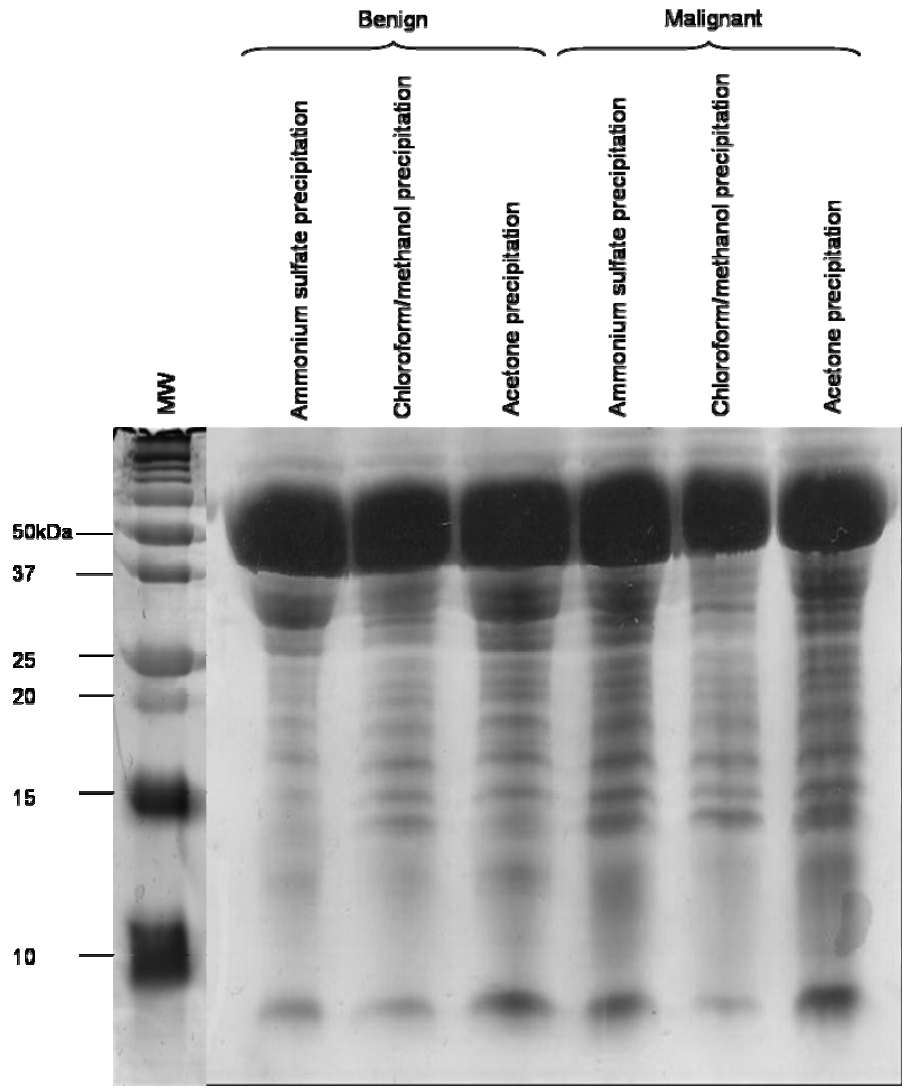


Figure 3.2 SDS-PAGE (12%) of cyst fluid proteins using three precipitation methods

Equal amount of cyst fluid proteins (10 μg) from representative benign and malignant epithelial ovarian tumours were loaded into each well. Three precipitation methods were utilised in this study as illustrated in the figure.

3.3.2 Protein fractionation and identification using SDS-PAGE and MALDI TOF-TOF/MS

Aim. The SELDI-TOF protein profiles are displayed according to their m/z ratio. Proteins with a single charge are separated based on their molecular weight. As mentioned in section 3.2, the presence of one candidate marker with the molecular weight approximately 17.5 kDa is indicated (Figure 3.1). Since the identity of this singly charged protein cannot be determined using the SELDI-TOF technique, conventional SDS-PAGE and MALDI TOF-TOF/MS methods were performed to separate and identify the target protein based on the molecular weight similarity.

Investigation. We utilised SDS-PAGE to separate the protein mixtures and isolate the corresponding protein bands between 15-20 kDa. SDS-PAGE separates proteins according to their size. Considering the molecular weight of the target protein, 15% SDS-PAGE was selected due to its ability to recover low molecular weight proteins. Cyst fluid proteins from benign and malignant epithelial ovarian tumours were prepared using acetone precipitation method (section 2.2.1.1) and silver stain (Bio-Rad Laboratory) was used to visualise the protein bands. Proteins of interest were detected either visually or with the aid of software (Bio-Rad Laboratory). The target gel band from SDS-PAGE (Figure 3.3) was excised and subjected to alkylation, reduction and protein digestion prior to identification by MALDI TOF-TOF/MS (section 2.2.9.2 and section 2.2.9.3).

Results. Figure 3.3 indicated that the protein bands between 15 kDa to 20 kDa were predominantly present in cyst fluids from late stage ovarian cancer samples when compared with benign ovarian tumours. Peptide mass fingerprinting using MALDI TOF/MS (section 2.2.9.2) followed by MASCOT search (section 2.2.9.4) identified the target protein as haptoglobin-α_2 subunit. The matched peptide

peaks were highlighted with arrows from the MASCOT search results (Figure 3.4 A). The sequence of matched peptides was noted to have 74% homology to that of haptolgobin-α_2 subunit (Figure 3.4 B). These results were significant with the p value being <0.05. The peptides with peak of high intensity were selected for MS/MS analysis. A summary of the sequence information is shown in Figure 3.4 C while the selected peptide sequences ascertained by MS/MS analysis are depicted in Figure 3.5.

Interpretations. Proteins in conventional SDS-PAGE are separated according to the molecular weight. The specific protein with molecular weight of 15-20 kDa identified in SDS-PAGE corresponded appropriately with that in the SELDI-TOF. This protein was observed to be significantly upregulated in late stage ovarian cancers (Figure 3.1 and 3.3). Using the MALDI TOF-TOF/MS technique it was noted the protein band was identified as haptoglobin-α_2 subunit. The identification also demonstrated a very high sequence coverage (74%) and Mowse scores (325), indicating the accurate determination of this protein. However, using SDS-PAGE, protein resolution was limited since a number of proteins share the similar molecular weights and comigrate to the same position in the gels (Figure 3.3). Since not all tryptic peptides from the selected protein spots were used for MS/MS analysis, the MS-based identification provided only an indication of haptolgobin-α_2 subunit. Alternative techniques are therefore required to confirm the identity of this molecule for subsequent clinical application.

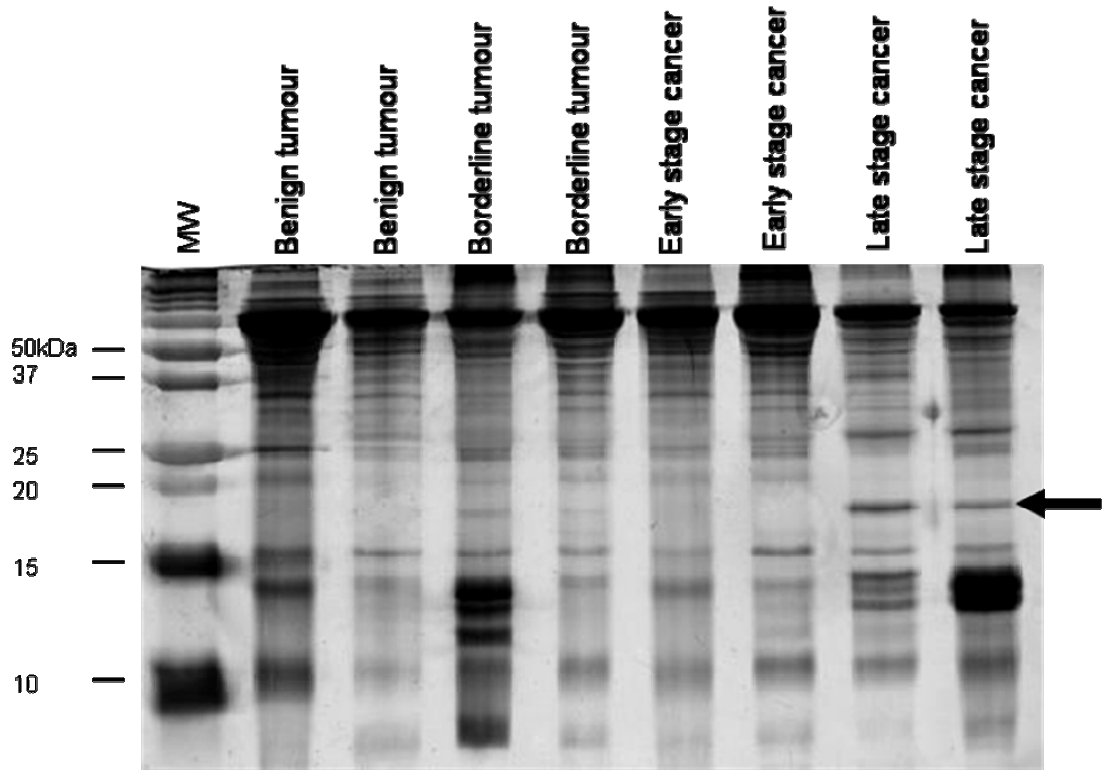
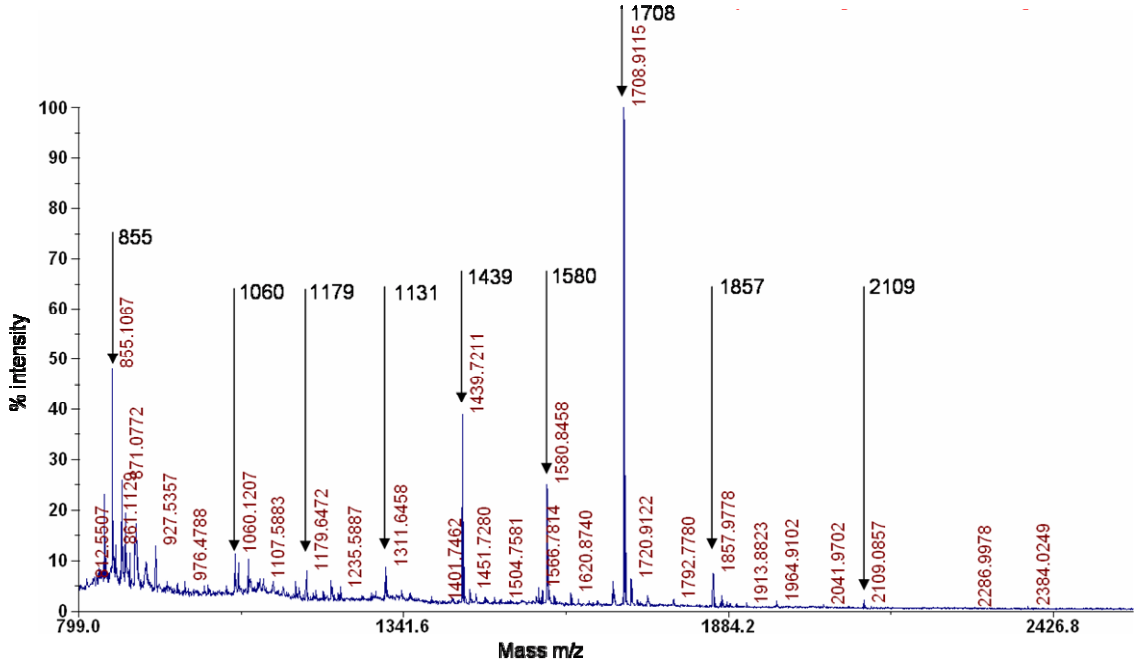


Figure 3.3 One-dimensional gel electrophoresis of representative cyst fluid proteins

Representative protein samples were obtained from benign ovarian tumour, borderline ovarian tumour, early stage and late stage ovarian cancers. The protein band indicated between 15-20 kDa was the corresponding unique protein with a molecular weight of 17.5 kDa peak obtained in Figure 3.1.

A



B

VNDSGNDVTD IADDGQPPPK CIAHGYVEHS VRYQCK**NYYK LRTQGDGVYT LNNEKQWINK AVGDKLPECE ADDGQPPPKC IAHGYVEHSV RYQCKNYYKL RTQGDGVYTL NNEKQWINKA VGDKLPECEA VGKPKNPANP VQ**

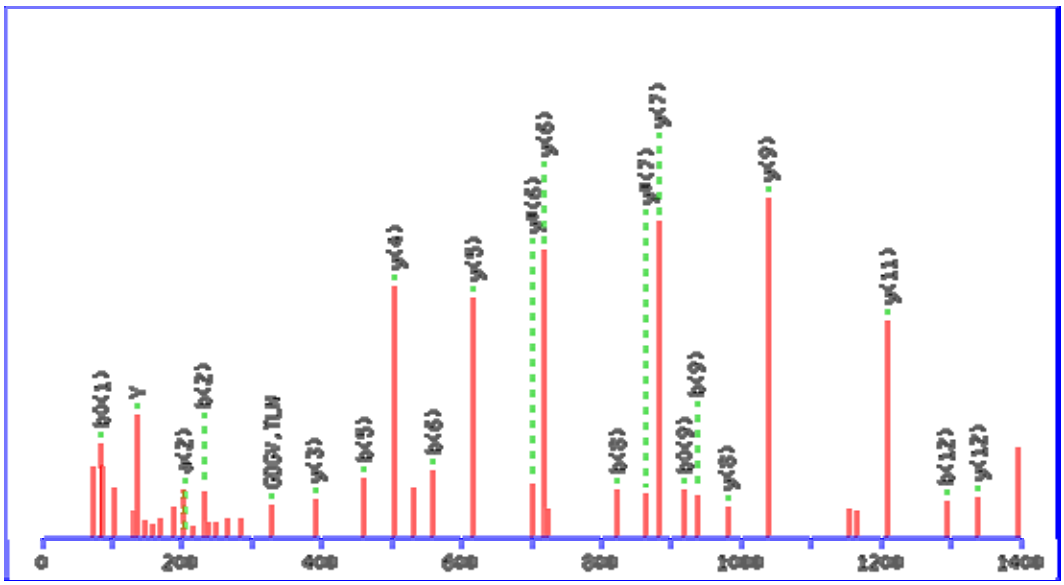
C

Observed	Mr(expt)	Start	End	Ions	Peptide
1439.72	1438.71	60 -	72	106	TEGDGVYTLNDKK
1580.85	1579.84	58 -	71	17	LRTEGDGVYTLNDK
1708.91	1707.90	117 -	131	49	LRTEGDGVYTLNNEK
1857.98	1856.97	137 -	153	29	AVGDKLPECEAVCGKPK

Figure 3.4 Mass spectrum generated by the 15~20 kDa protein band from SDS-PAGE using MALDI TOF/MS

A: PMF from the 15~20 kDa protein band. Arrows at the mass spectrum peaks represent peptides from haptoglobin-α_2 subunit. B: Sequence of human haptoglobin-α_2 subunit (NCBI database accession No. 701184A) and the matched peptide sequence (bold). Sequence coverage: 74%. C: The peptide sequence from four most intense peaks observed in the mass spectra using MS/MS analysis.

MS/MS Fragmentation of **TEGDGVYTLNNEK**



MS/MS Fragmentation of **LRTEGDGVYTLND**

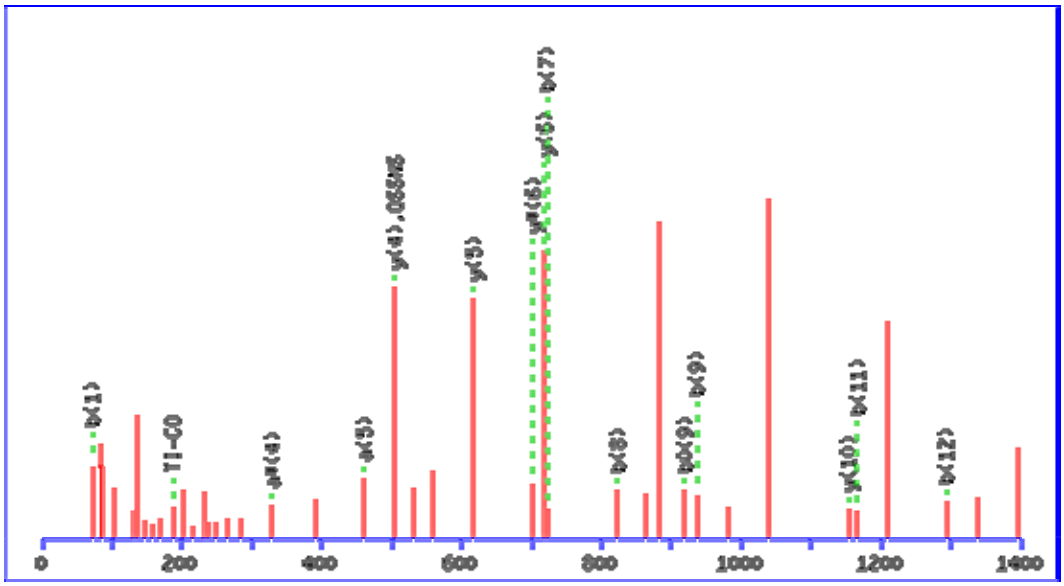


Figure 3.5 Representative CID generated MS/MS spectrum of the amino acid peptide sequence

Amino acid sequence of **TEGDGVYTLNNEK** [$(M+H)^+$ m/z 1439.88] and **LRTEGDGVYTLND** [$(M+H)^+$ m/z 1581.02] from haptoglobin-α_2 subunit are shown. Only b-, y- and a-type fragment ions are produced that can be used to confirm the amino acid assignments.

3.4 Validation of haptoglobin-α_2 subunit using PS20 immunocapture proteinChip analysis

Aim. In previous section, we assumed the protein band between 15-20 kDa in SDS-PAGE to be the corresponding protein peak in SELDI-TOF experiment based on the similarity of molecular weight (Figure 3.1 and 3.3). In this section, immunocapture proteinChip experiment was utilised to further confirm that the predominantly present protein peak in Figure 3.1 was haptoglobin subunit.

Investigation. PS20 proteinChip is used to covalently immobilised biomolecules for the subsequent capture of proteins from complex biological samples and well suited for antibody-antigen interaction study (ProteinChip System Users Guide, Ciphergen). Immunocapture experiments were performed with this proteinChip preincubated with rabbit anti-human haptoglobin antibody. Experiment procedure control was carried out using proteinChip preincubated with TNF-α antibody. Purified haptoglobin antigen was used as internal positive control and IgG as negative control. Cyst fluid proteins from selected benign and malignant tumours were analysed and data acquisition was carried out as described in section 2.2.4.

Results and interpretation. Results from this procedure indicated that malignant ovarian tumour had the same specific protein peak with the molecular weight around 17.5 kDa corresponding to haptoglobin-α_2 subunit, which was also observed in the positive control. Hence, this experiment provided confirmatory evidence that the predominantly present 17.5 kDa protein peak in late stage ovarian cancer using SELDI-TOF was haptoglobin-α_2 subunit (Figure 3.6).

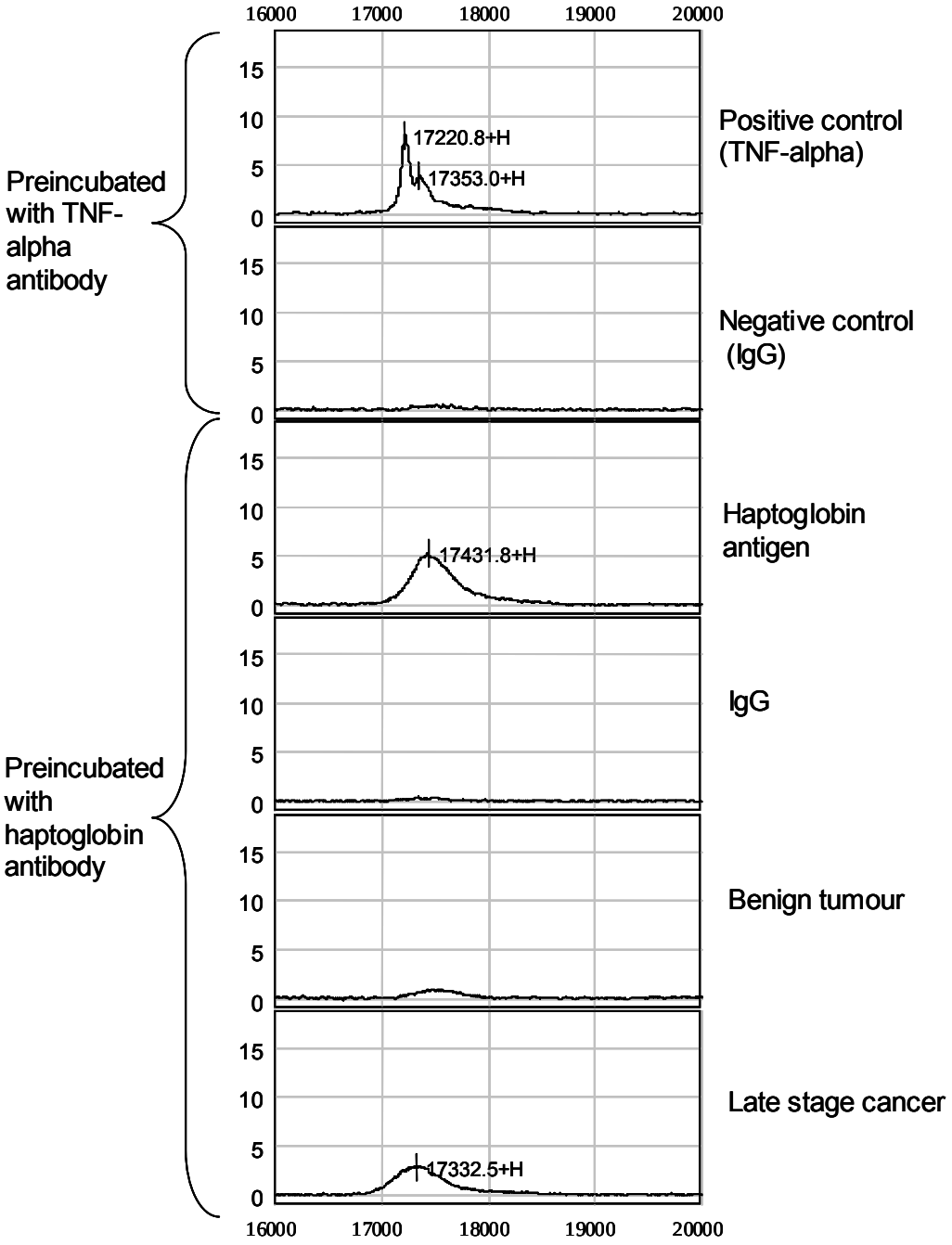


Figure 3.6 Immunocapture experiments using PS20 proteinChip

The immunocapture experiments were performed with PS20 proteinChips preincubated with anti-haptoglobin antibody to provide additional evidence confirming the presence of 17.5 kDa peak observed in Figure 3.1 to be haptoglobin-α_2 subunit. Haptoglobin antigen and Bovine IgG were used as the internal positive and negative control. Cyst fluid protein from late stage ovarian cancer and benign tumour were exposed to the antibody-coated arrays. TNF-alpha and Bovine IgG were used in chips preincubated with TNF-alpha antibody as procedure control.

3.5 Validation of haptoglobin-α_2 subunit as potential protein markers in EOC using conventional immunologic methods

3.5.1 Validation using Western blotting analysis

Aim. In order to provide additional evidence, traditional immunological method Western blotting was used to validate the aberrant expression of haptoglobin-α_2 subunit in cyst fluid from benign and malignant ovarian tumours.

Investigation. Cyst fluid proteins from benign and malignant ovarian tumours were subjected to 15% SDS-PAGE as described earlier (section 2.2.5). Polyclonal rabbit anti-human haptoglobin antibody was used for Western blotting analysis (section 2.2.10). Band intensities from different groups were measured using Quantity One® software (Bio-Rad Laboratories)

Results and interpretation. Results from the blotting procedure indicated the presence of different subunits of haptoglobin in cyst fluid from epithelial ovarian tumours as shown in Figure 3.7. Based on the molecular weight, the corresponding subunits identified were β (40 kDa), α_2 (17.5 kDa) and α_1 (9 kDa) subunits. The haptoglobin-α_2 subunit was the protein of interest identified from SELDI-TOF and SDS-PAGE procedures. Combined with immunocapture result, this data provided confirmatory evidence that haptoglobin-α_2 subunit was elevated in cyst fluid from EOC when compared with benign disease. It was also observed that the intensities of other haptoglobin subunits were increased in cyst fluid from malignant ovarian tumours.

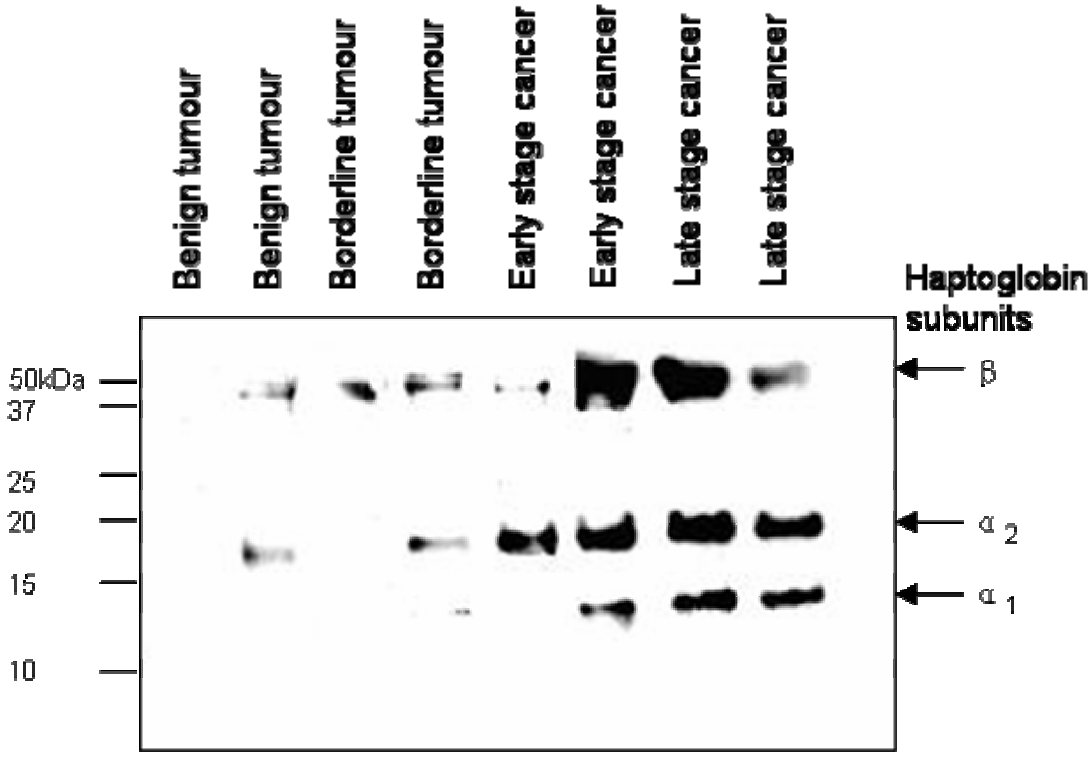


Figure 3.7 Confirmation of haptoglobin using Western blotting analysis
Confirmation of haptoglobin was performed using polyclonal rabbit anti-human haptoglobin antibody to detect the different subunits of this protein. Equal amounts of protein (10 µg) from benign, borderline ovarian tumour, early stage and late stage ovarian cancer were loaded into the 15% SDS-PAGE. Based on the molecular weight, the corresponding subunits identified were β (40 kDa); α_2 (17.5 kDa) and α_1 (9 kDa). High intensity bands were distinctly present in samples from early and late stage ovarian cancers when compared with benign ovarian tumours.

3.5.2 Generation of polyclonal antibody and quantitative analysis of haptoglobin-α subunit in cyst fluid using in-house established ELISA method

Aim. The aberrant distribution of haptoglobin-α subunit in benign and malignant ovarian tumours has been verified using semi-quantitative methods such as SELDI-TOF, SDS-PAGE and Western blotting (section 3.2, 3.3, 3.5.1). In order to obtain the absolute quantity of this protein of interest in cyst fluid, we proposed to develop the in-house sandwich ELISA method using two antibodies, one of which should bind haptoglobin-α subunit specifically. So far there is no commercially available antibody specifically against haptoglobin-α subunit. Therefore, a polyclonal rabbit anti-human haptoglobin-α subunit antibody was to be generated.

Investigation. The polyclonal rabbit anti-human haptoglobin-α antibody was generated as described in section 2.2.11. Goat anti-human haptoglobin antibody (Biodesign) and the generated antibody were used in this ELISA experiment as described in section 2.2.12. The precision of the detection method for haptoglobin-α subunit antigen was evaluated using intra-and inter-assay coefficients of variation (CV). The intra-assay coefficient of variation was revealed by choosing three haptoglobin-α subunit antigen-positive cyst fluid samples by parallel determination of 8 wells simultaneously each. The inter-assay coefficient of variation was demonstrated by determination of the above samples for 5 consecutive days.

Results and interpretation. The CVs were 4.5% and 13.2% for the intra-assay and inter-assays respectively, which reached the requirement for intra and inter-assay CVs for diagnostic kits (Chen et al., 1999). Comparison of the mean of haptoglobin-α subunit levels in benign subjects and those presenting with early and late stage cancers was analysed using the ANOVA analysis. A significant

difference between the three groups of patients was observed as indicated in Table 3.2 and Figure 3.8.

Table 3.2 Mean concentration of haptoglobin-α subunit in benign and malignant ovarian tumours by the ELISA procedure

Group	Haptoglobin-α subunit (μg/ml)		
	No.	Mean	P
Late Cancer	17	216.3	<0.01
Early and borderline	11	137.9	
Benign tumour	35	71.4	

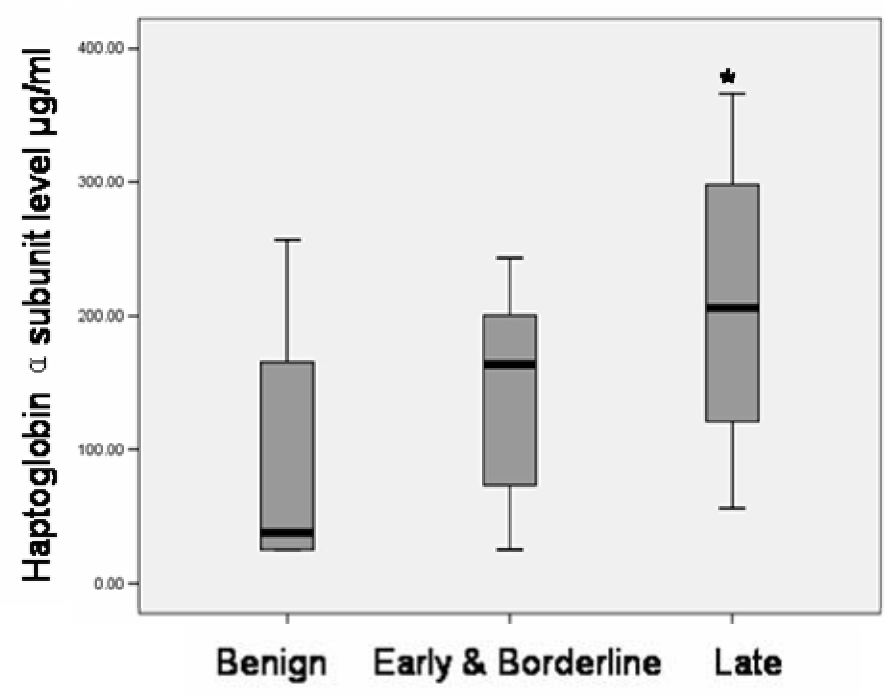


Figure 3.8 Concentrations distribution of cyst fluid haptoglobin-α subunit
The concentrations of cyst fluid haptoglobin-α subunit obtained from 17 patients with EOC, 11 borderline tumour and 35 benign ovarian tumours were demonstrated. The haptoglobin-α subunit levels were significantly higher in patients with late stage of ovarian cancer when compared to those with benign tumours (*P<0.01).

3.6 Conclusion

SELDI-TOF mass spectrometry technology offers a powerful and sensitive way to study post-translational protein profiles. Previous study (Yang et al., 2005) already showed the effectiveness using SELDI-TOF coupled with gel based fractionation and MS based identification methods in identification of brain derived neurotrophic factor to be a novel functional protein in development of hepatocellular carcinoma. In this study, by proteinChip technique, we monitored an upregulated cyst fluid proteins in malignant when compared with benign ovarian tumours, indicating that this protein might be closely related to tumour pathogenesis and had the potential to be a candidate biomarker for this insidious cancer. We then identified this protein from malignant cyst fluid using SDS-PAGE and MS to be haptoglobin-α_2 subunit. Using specific immunocapture experiment and antibody generated against this subunit, the expression pattern of haptoglobin-α subunit was confirmed. Hence, our study suggests that the technical triads starting with (1) SELDI-TOF proteinChip technology, (2) SDS-PAGE to separate proteins of interest based on molecular weight, followed by (3) in-gel digestion of selected protein bands, (4) identification of proteins using MALDI TOF-TOF tandem MS and (5) series of validation methods including immunocapture proteinChip, Western blotting and ELISA methods are ideal models in searching for potential protein markers for EOC. The upregulated haptoglobin-α_2 subunit could be a useful biomarker for EOC.

Chapter 4: 2-DE based identification of differentially expressed cyst fluid protein biomarkers for EOC

4.1 Introduction

In the preceding chapter, the identification of haptoglobin-α_2 subunit as a potential biomarker for EOC using SELDI-TOF based methods was described. This technique, however, is only suitable for the recovery of relatively low molecular weight proteins (Petricoin et al., 2004). Hence, SELDI-TOF-based method used in the investigation limits the ability to isolate suitable protein biomarkers which may be identified by alternative methods. In this regard, 2-DE was chosen to be a complementary technique to the SELDI-TOF with a view to identify other protein biomarkers of interest for the diagnosis or prognosis of ovarian cancer.

2-DE consisting of IEF and SDS-PAGE eables the separation of complex protein mixtures according to p*I* and molecular weight. It can resolve more than 5000 proteins simultaneously and is suitable for both high and low molecular weight proteins (Gorg et al., 2004). Moreover, the 2-DE technique has the capability to identify proteins that have undergone post translational modification including phosphorylation, glycosylation or limited proteolysis. The modified proteins in 2-DE gels can be readily located as they appear as distinct spot trains in the horizontal and/or vertical axis of the gel (Gorg et al., 2004). In this context, it has been reported that differential expression of haptoglobin precursor, transferrin, cSHMT, Tbx3 and RhoGDI identified using 2-DE occurred in serum, plasma and

tissues from ovarian tumour patients (Jones et al., 2002; Ahmed et al., 2004; Lomnytska et al., 2006).

Based on this premise, a comprehensive study using 2-DE coupled with MALDI-TOF and traditional immunological validation methods was performed in a bid to further characterise alternative biomarkers. Attempts were made to verify the hypothesis 1 "Differentially expressed proteins may be present in cyst fluid from benign and malignant ovarian tumours and could be identified using 2-DE and traditional immunological technology".

4.2 Protein sample preparation prior to 2-DE

Aim. Several high abundance proteins such as albumin and immunoglobin G in human serum may dominate the protein profile and mask potential low abundance biomarkers. In human plasma, albumin accounts for 57–71% (w/w) of the total protein and is present at concentrations of 35–50 mg/ml, while the potential protein markers are always present in µg or pg ranges (Anderson et al., 2002). Moreover, uneven distribution of protein concentrations in serum could result in horizontal or vertical smears on the 2-DE, which poses a difficulty in image analysis (Magnani et al., 2004). One approach to facilitate biomarker discovery would be to prefractionate body fluids using antibody chelating columns to remove high abundance proteins, thereby increasing the potential for analysing and detecting useful biomolecules.

Investigation. Protein composition in cyst fluid was studied using SDS-PAGE (section 2.2.5). In order to remove the high abundance protein albumin, cyst fluid samples were subjected to affinity column purification using Aurum Serum Protein Mini kit (Bio-Rad laboratories; section 2.2.8.1). To evaluate if albumin

was successfully removed in the samples, an aliquot of fractionated and unfractionated cyst fluid (10 µl) were subjected to SDS-PAGE and 2-DE (section 2.2.5 and section 2.2.8) followed by visualisation with silver stain (Biorad laboratory).

Results and interpretation. As shown in Figure 4.1 and 4.2, using SDS-PAGE and MALDI TOF-TOF/MS it was observed that albumin comprised the major portion of cyst fluid proteins. Figure 4.3 indicated that the fractionated cyst fluid sample in which the albumin was substantially removed had a better resolution of the protein bands when compared with the unfractionated samples. Silver staining of 2-DE from albumin depleted samples also revealed the presence of additional protein spots with low molecular weight which could be readily identified and characterised (Figure 4.4). Therefore, this Aurum Serum Protein Mini column provides a simple, rapid and effective method for depletion of albumin from human cyst fluid samples.

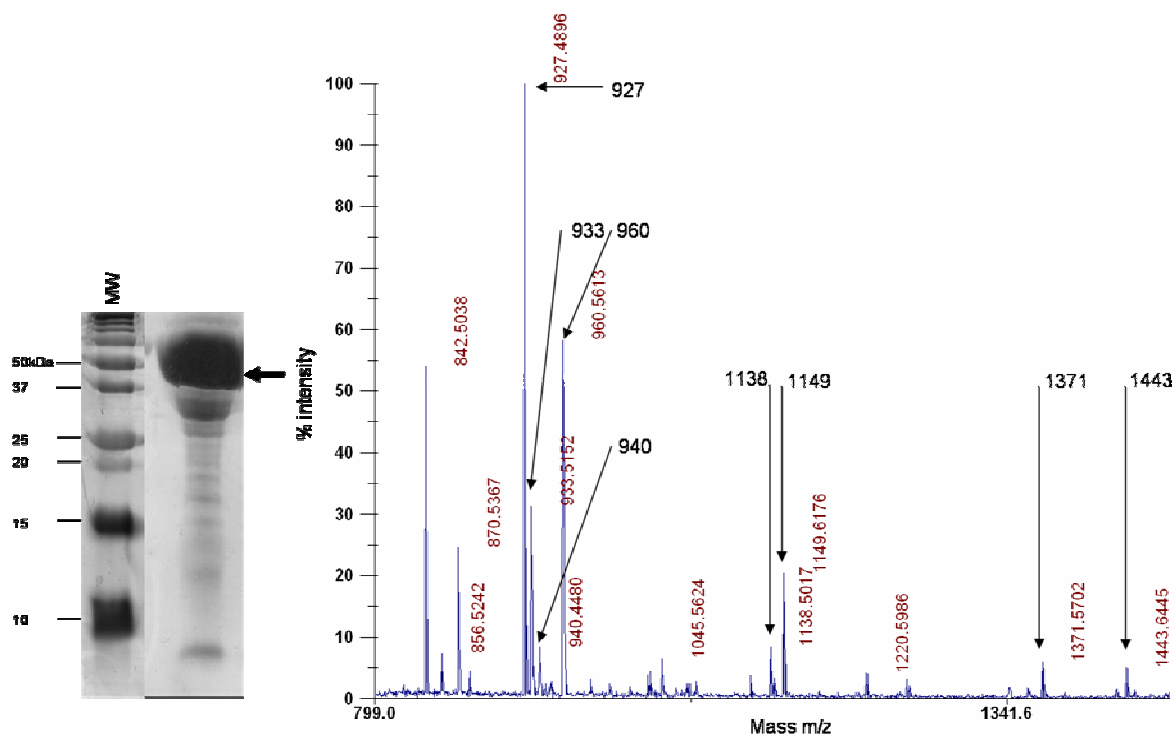
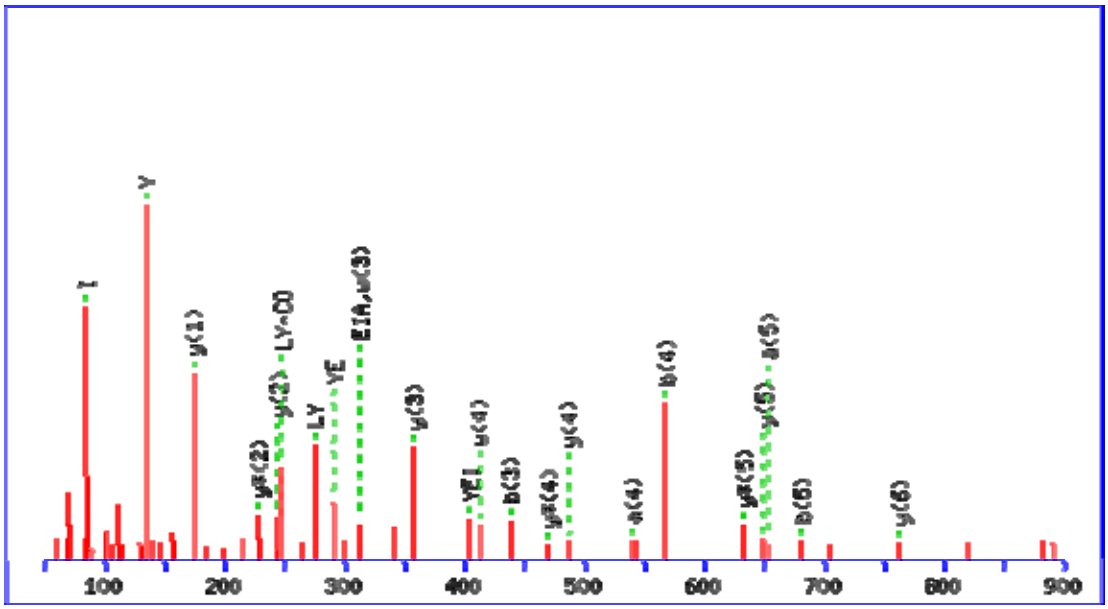


Figure 4.1 Identification of the high abundance protein in cyst fluid as albumin

The protein band indicated in the left panel (arrowed) was subjected to MALDI TOF/MS analysis. Mass spectrum analysis generated by this high abundance protein using MALDI TOF/MS is shown in the right panel. Arrows at the mass spectral peaks represent peptides from albumin.

MS/MS Fragmentation of **YLYEIAR** (score 27)



MS/MS Fragmentation of **DDNPNLPR** (score 41)

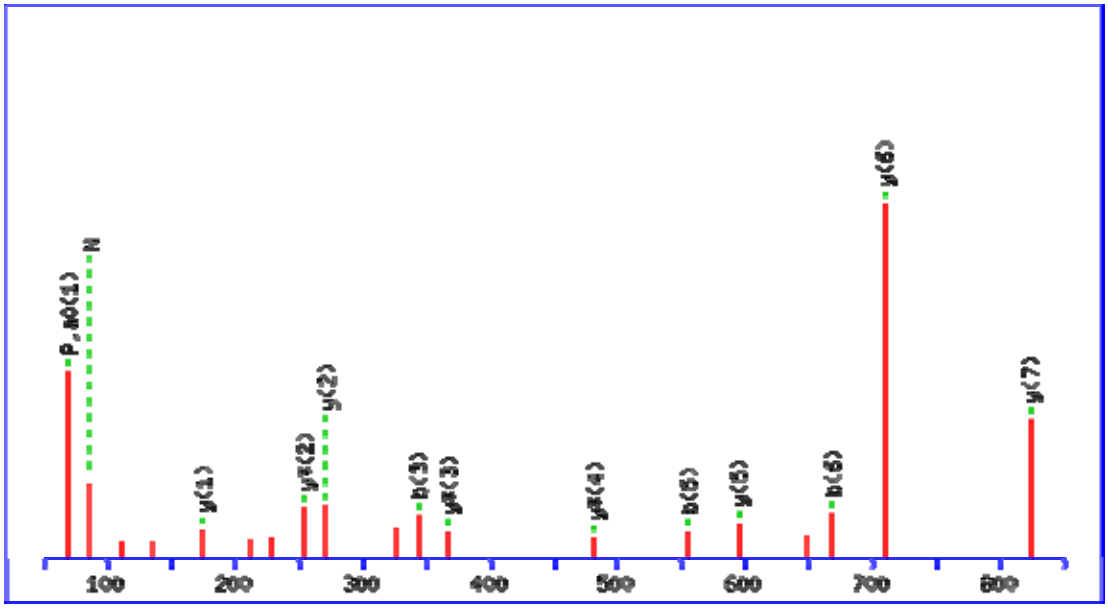


Figure 4.2 Representative CID generated MS/MS spectrum of the amino acid peptide sequence

Amino acid sequences of **YLYEIAR** [$(M+H)^+$ m/z 927.51] and **DDNPNLPR** [$(M+H)^+$ m/z 940.47] from albumin are shown. Only b-, y- and a-type fragment ions are produced that can be used to confirm the amino acid assignments.

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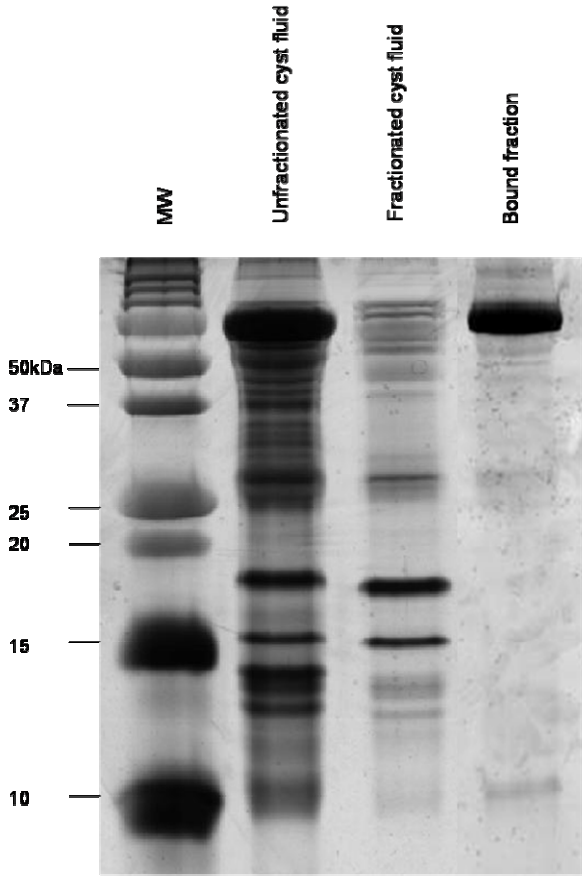


Figure 4.3 SDS-PAGE analysis of representative cyst fluid protein sample before and after albumin depletion using the anti-albumin antibody chelating column

Unfractionated cyst fluid (proteins without albumin depletion), the fractionated cyst fluid (proteins following Aurum Serum Protein Mini column purification) and bound fraction (proteins recovered from the membrane) are indicated respectively. It was observed that the albumin depleted samples of cyst fluid resulted in a better resolution of the protein bands when compared with the unfractionated samples.

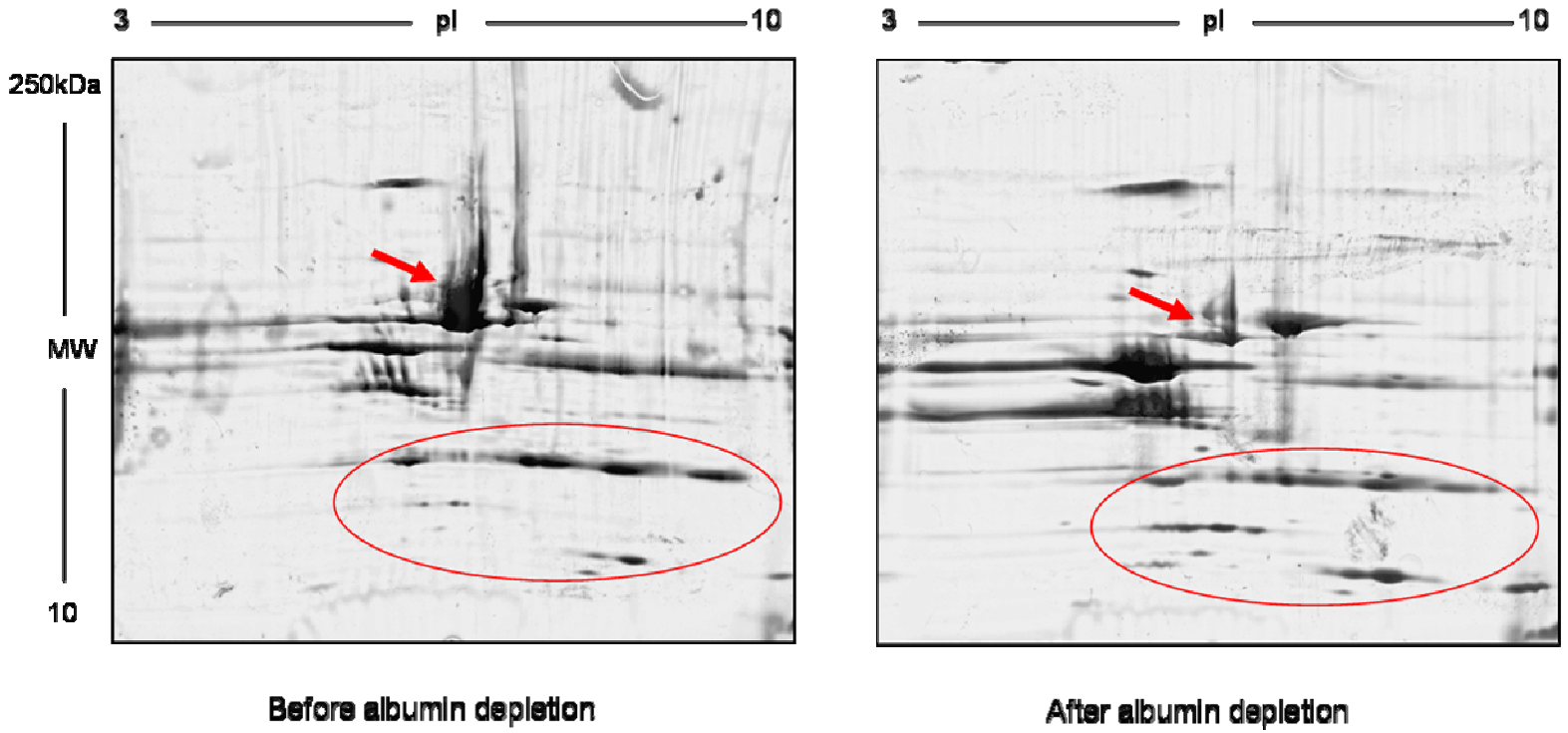


Figure 4.4 2-DE analysis of representative cyst fluid protein sample before and after albumin depletion

Cyst fluid proteins were analysed using pH three to ten IPG strip for the first dimension and 4-20% gradient SDS-PAGE for the second dimension after silver staining. Arrows showed the effectiveness of albumin depletion and the presence of additional protein spots in the low molecular weight regions were also highlighted as circles.

4.3 Identification of differentially expressed proteins in cyst fluid using 2-DE based proteomics methods

4.3.1 Cyst fluid protein profiles from benign and malignant epithelial ovarian tumours using 2-DE

Aim. To examine the protein profiles and detect differentially expressed proteins in cyst fluid from EOCs using 2-DE

Investigation. For 2-DE analysis, cyst fluids from benign (n=10), borderline tumours (n=7), early (n=3) and late stage (n=12) EOCs were analysed in triplicate as mentioned in section 2.2.8. Following removal of high abundance proteins including albumin, the cyst fluid samples were subjected to quantification using the RC DC protein assay (Bio-Rad laboratories). Briefly, for the 2-DE procedure, 100 µg of cyst fluid protein was mixed with 185 µl of rehydration buffer, subjected to electrophoresis in IPG strips of 11 cm at a pH of 4–7 for the first dimensional separation and to a 4-20% tris-HCl criterion gel (Bio-Rad Laboratories) for the second dimensional separation. Protein spots were visualised by silver staining and spot detection, quantification and matching were performed using the PDQuest version 7.2 software package (Bio-Rad Laboratories).

Results and interpretation. Representative cyst fluid protein profiles from benign, borderline tumour, early and late stage ovarian cancers performed by this procedure are presented in Figure 4.5. An average of 160-180 spots for each sample was detected in the gels at the pH range 4-7 (Figure 4.5). It was observed that four regions of significant and consistent up-regulated spots with an intensity change of more than two-fold were detected with the PDQuest software (illustrated in Figure 4.5 and 4.6) from malignant EOC cases when

compared with benign tumours. As indicated in Figure 4.6, representative samples of up-regulated spots in region A could be observed in two early, four borderline and seven late stage ovarian cancers; two early, two borderline and five late ovarian cancer were noted for spots in region B; one early, two borderline and five late ovarian cancer for region C and one early, three borderline and six late ovarian cancer for region D after computing for volume intensity difference between benign and malignant ovarian tumours. Data from this 2-DE technique also indicated the presence of isoforms for the above proteins as indicated by multiple protein spots with different molecular mass and p*I*s, suggesting possible co- or post-translational modifications. It was also observed that a significant heterogeneity in protein patterns occurred in these cyst fluid samples. All significant protein spots which were reproducible by the 2-DE technique were excised from the gels and subjected to MALDI TOF-TOF/MS analysis.

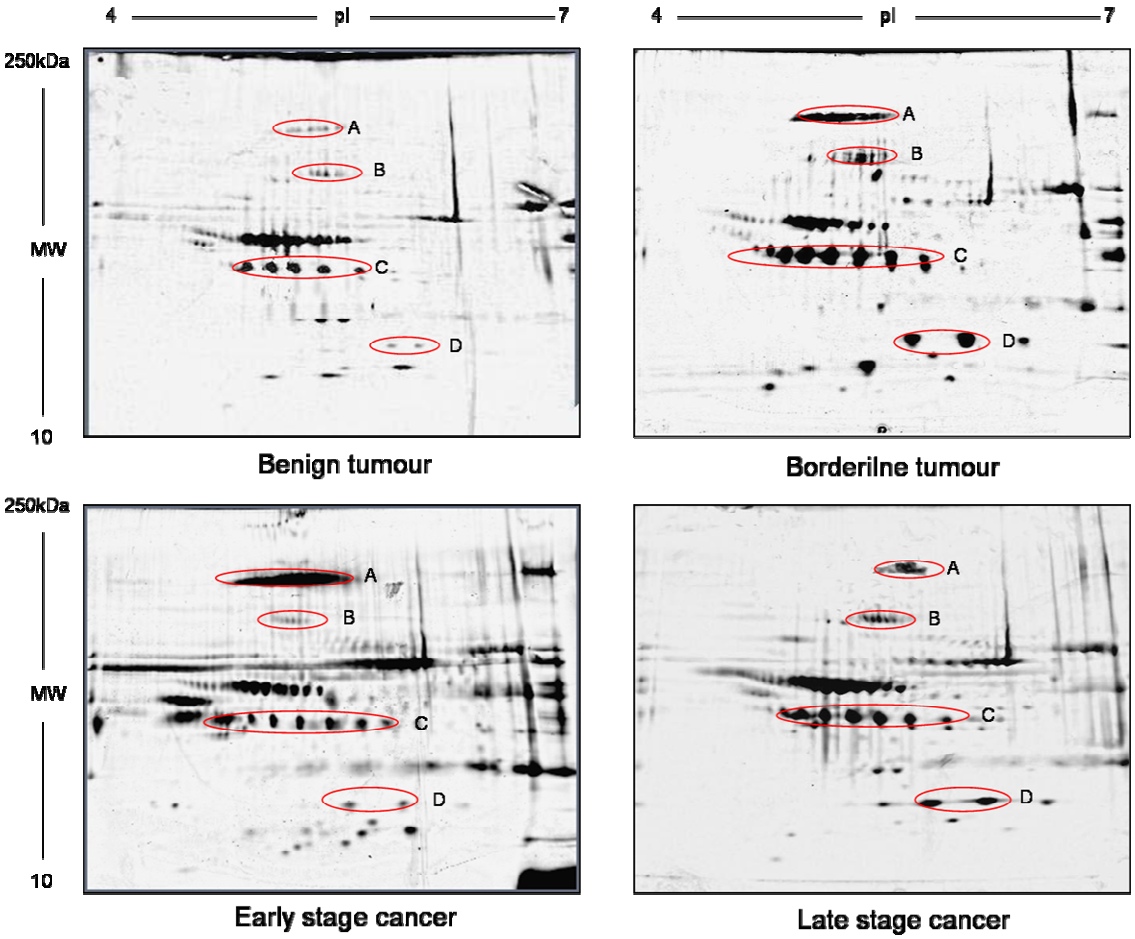


Figure 4.5 Representative silver-stained 2-DE of cyst fluid protein profiles from benign, borderline tumours as well as early and late stage ovarian cancers

50ng of protein was used for each experiment and the staing time for each sample was identical. The differences in spot intensities could be visually appreciated and identified using PDQuest software. Four areas were identified and the consistent spot changes observed were represented as circles. Molecular weight markers were indicated on the left (in kDa) and the corresponding isoelectric point (p*I*) was indicated across the top of the gels.

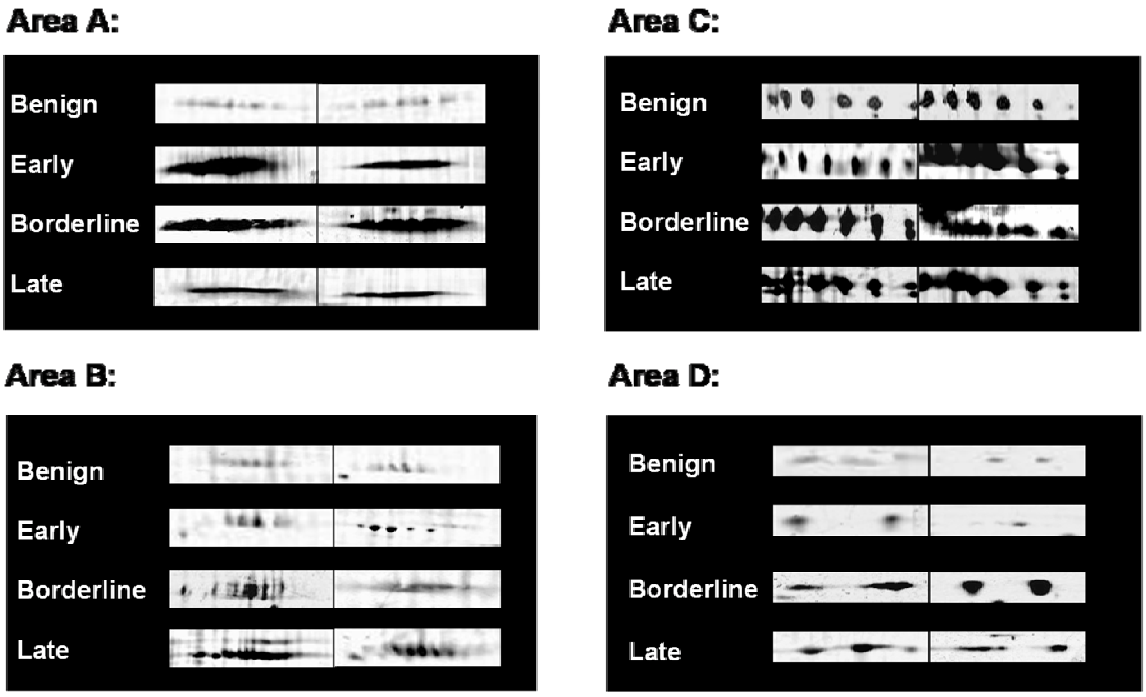


Figure 4.6 Elevated expression of protein spots in cyst fluid from malignant compared to benign ovarian tumours

Image analysis was performed using PDQuest software. Results from two benign; two borderline tumours; two early and two late stage ovarian cancers are presented. These are representative of ten benign tumours, seven borderline tumours, three early and twelve late stage ovarian cancers.

4.3.2 Identification of differentially expressed proteins in malignant ovarian tumours using MALDI TOF-TOF/MS

Aim. To identify the differential protein spots in 2-DE from malignant cyst fluid mentioned in section 4.3.1

Investigation. The differentially expressed protein spots in 2-DE gels (Figures 4.7) from malignant ovarian cyst fluids were excised and the corresponding peptides were eluted by in-gel digestion. MALDI TOF-TOF/MS was employed for identification of these protein spots (section 2.2.9)

Results and interpretation. MALDI TOF-TOF/MS (section 2.2.9.2) followed by MASCOT search (section 2.2.9.4) identified the proteins of interest circled in Figure 4.5 as ceruloplasmin and haptoglobin. Matched peptide peaks from MASCOT search are highlighted (Figure 4.8 and 4.10, arrows). The sequence of matched peptides has 28% and 45% homology to that of ceruloplasmin and haptoglobin respectively. A summary of the peptide sequence information is shown in Figure 4.8 B and 4.10 B while the representative peptide sequences ascertained by MS/MS analysis are depicted in Figure 4.9 and 4.11. Other proteins identified from the selected spots with their matched peptides, sequence coverage, MOWSE score, theoretical molecular weight and p*I* are listed in Table 4.1. Ceruloplasmin and haptoglobin were further analysed by Western blotting, since they were consistently and significantly altered in cyst fluid from EOC when compared with benign ovarian tumours. Identification of candidate markers in the 60 kDa-120 kDa molecular weight regions provides evidence for hypothesis 1 in which it was proposed that 2-DE could be a complementary method to SELDI-TOF in biomarker identification for EOCs.

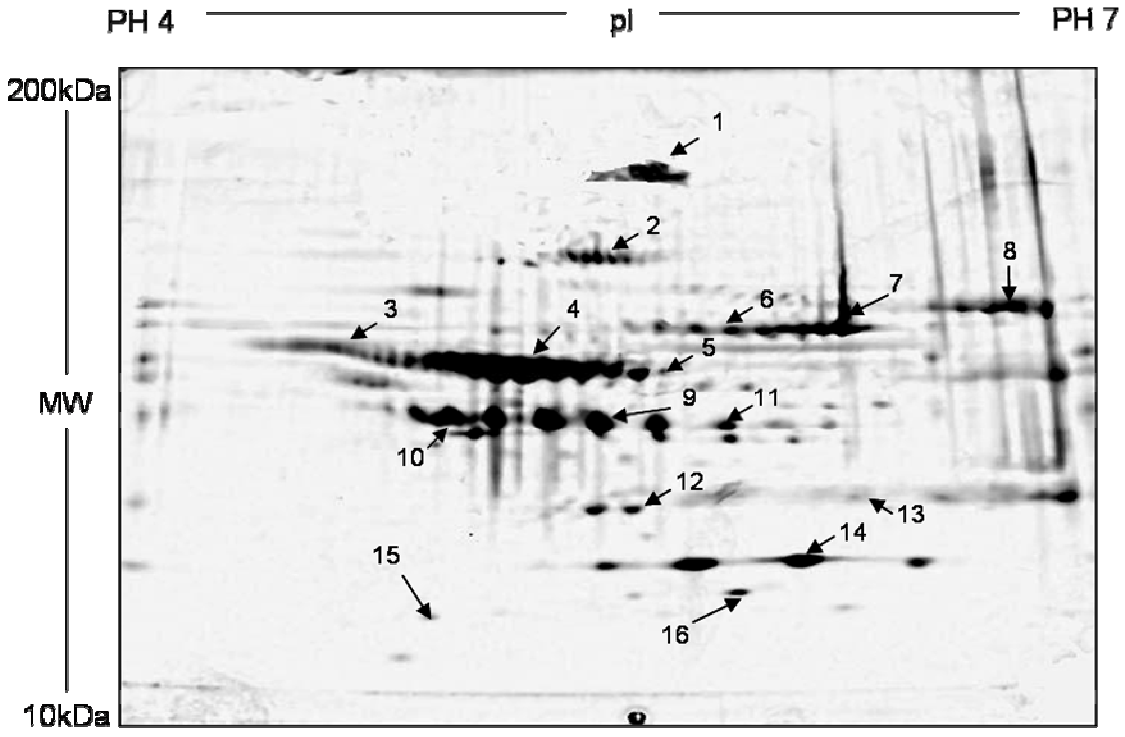
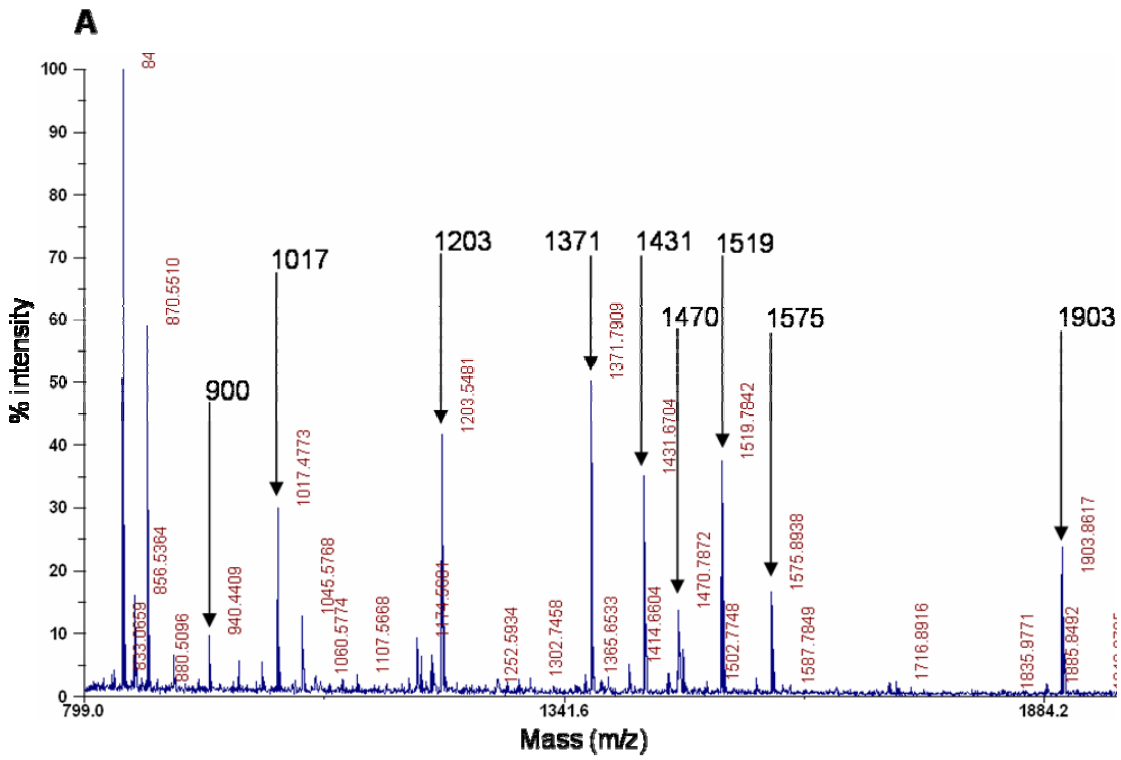


Figure 4.7 A representative map of silver-stained 2-DE profile obtained from cyst fluid of EOC.

The protein spots which have been identified are listed in Table 4.1.



B

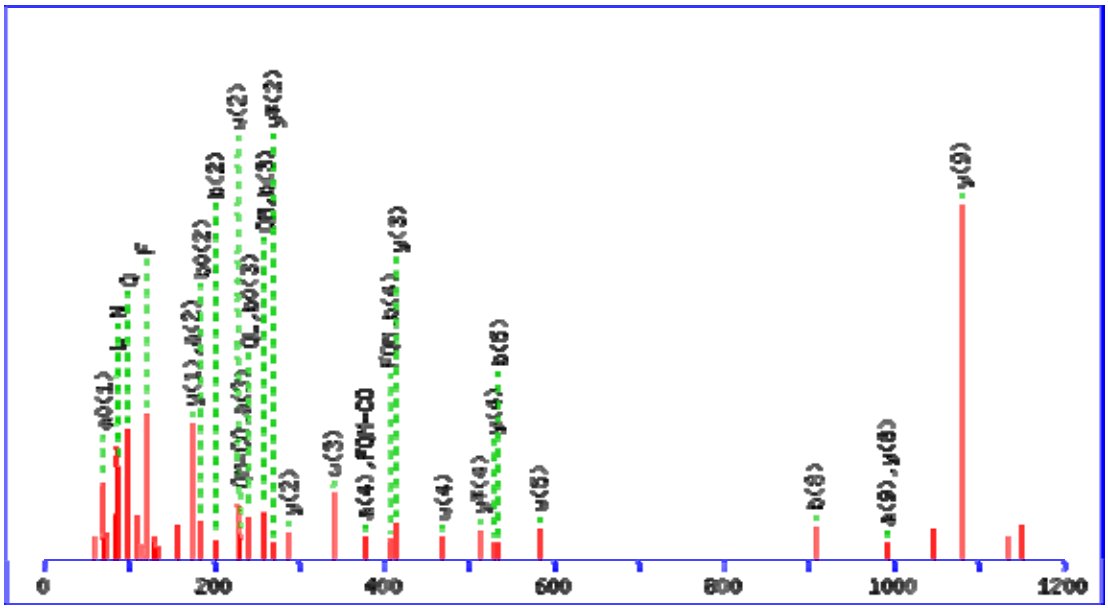
Observed	Mr(expt)	Start	End	Ions	Peptide
1519.78	1518.78	70	81	60	ALYLQYTDETFR
1203.55	1202.54	427	436	42	EYTDASFTNR
1371.79	1370.78	469	481	53	GAYPLSIEPIGVR
1903.86	1902.85	485	500	24	NNEGTYYSPNYNPQSR
1470.79	1469.78	524	537	32	EVGPTNADPVCLAK
1431.67	1430.66	721	732	67	QSEDSTFYLGER
1017.48	1016.47	786	793	33	QYTDSTFR

Figure 4.8 Identification of ceruloplasmin using MALDI TOF-TOF/MS

A: Mass spectrum generated by the protein No 1 using MALDI-TOF/MS. Arrows at the peaks of mass spectra represent peptides from ceruloplasmin

B: The peptide sequence from the most intense peaks observed in the mass spectra using MS/MS analysis

MS/MS Fragmentation of **EYTDASFTNR** (ion score 42)



MS/MS Fragmentation of **QYTDSTFR** (ion score 33)

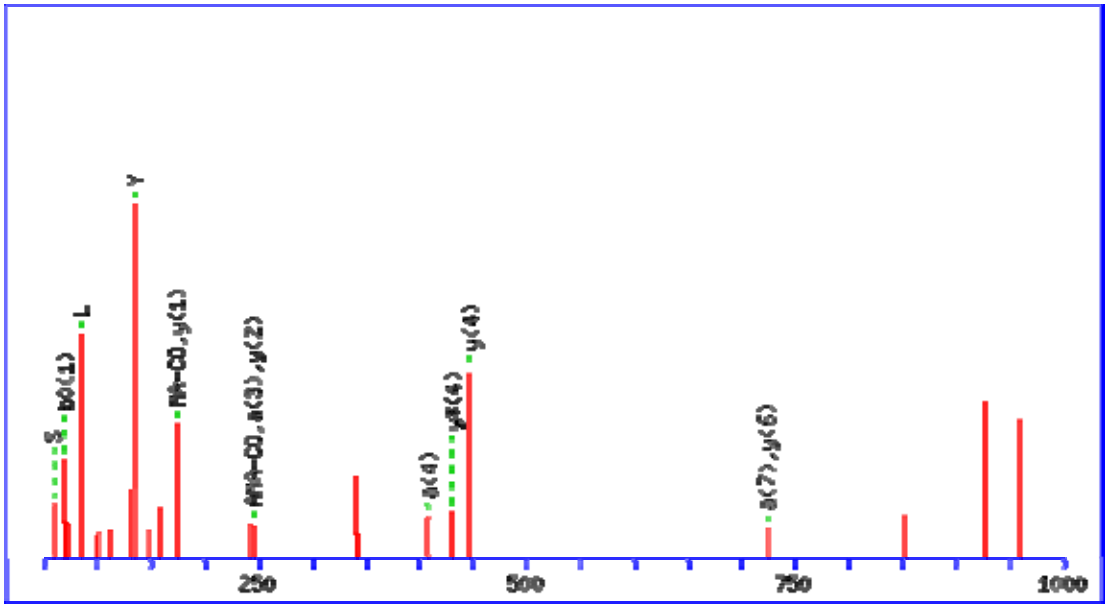
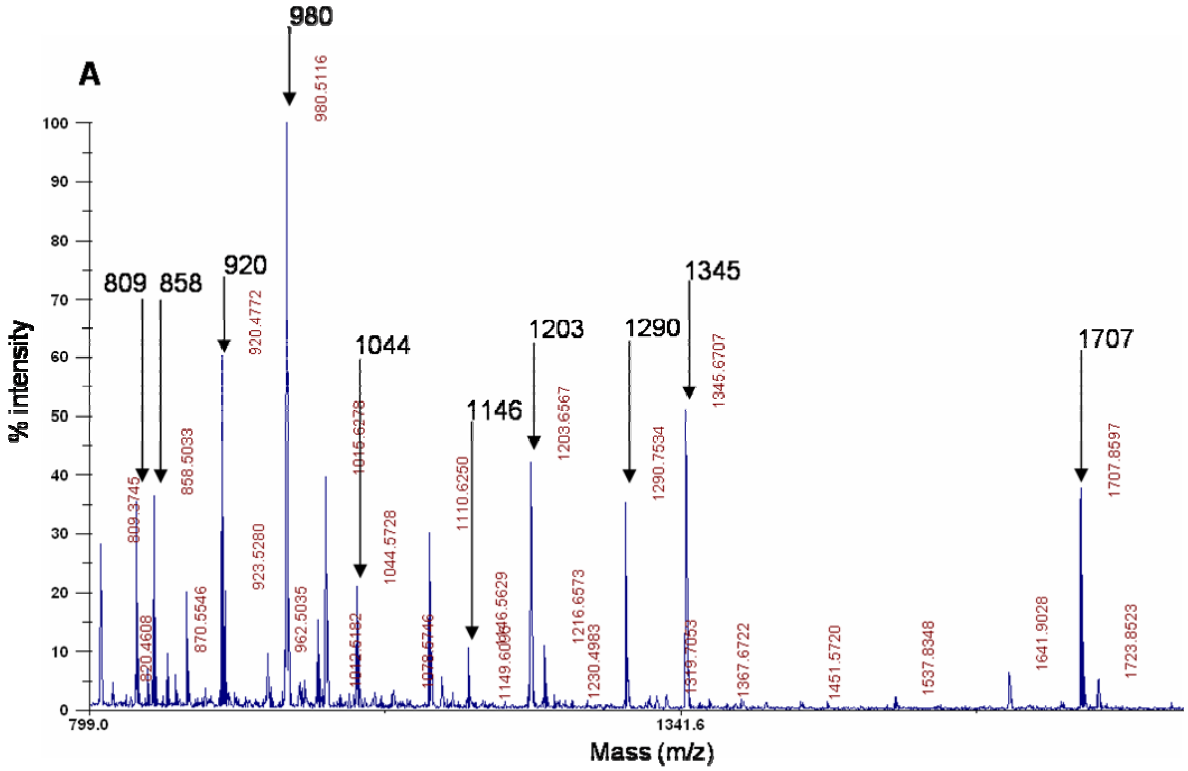


Figure 4.9 Representative CID generated MS/MS spectrum of the amino acid peptide sequence

Amino acid sequence of **EYTDASFTNR** [$(M+H)^+$ m/z 1203.55] and **QYTDSTFR** [$(M+H)^+$ m/z 1017.48] from ceruloplasmin are shown. Only b-, y- and a-type fragment ions are produced that can be used to confirm the amino acid assignments.



B

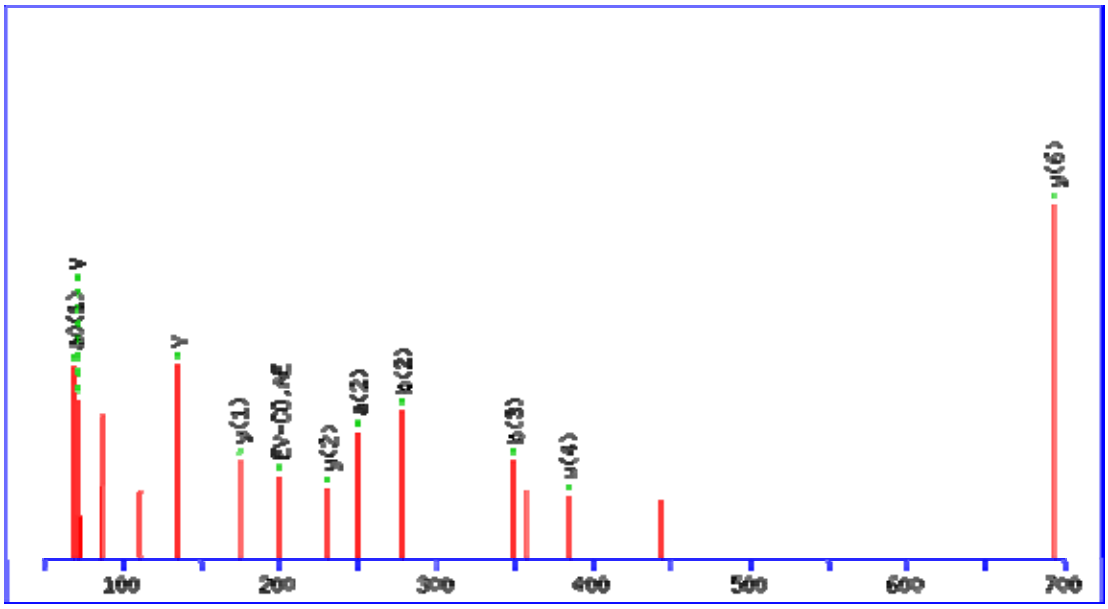
observed	Mr(expt)	Start	End	Ions	Peptide
809.37	808.37	146	152	39	DYAEVGR
858.50	857.50	104	110	43	QLVEIEK
920.48	919.47	46	53	22	GSFPWQAK
980.51	979.50	153	161	62	VGYVSGWGR
1203.66	1202.65	267	276	69	VTSIQDWVQK
1290.75	1289.75	91	102	69	DIAPTLTLYVGK
1345.67	1344.66	255	266	112	SCAVAEYGVYVK

Figure 4.10 Identification of haptoglobin using MALDI TOF-TOF/MS

A: Mass spectrum generated by the protein spot No 9 using MALDI-TOF/MS. Arrows at the mass spectrum peaks represent peptides from haptoglobin.

B: The peptide sequence from seven most intense peaks observed in the mass spectra using MS/MS analysis.

MS/MS Fragmentation of **DYAEVGR**



MS/MS Fragmentation of **GSFPWQAK**

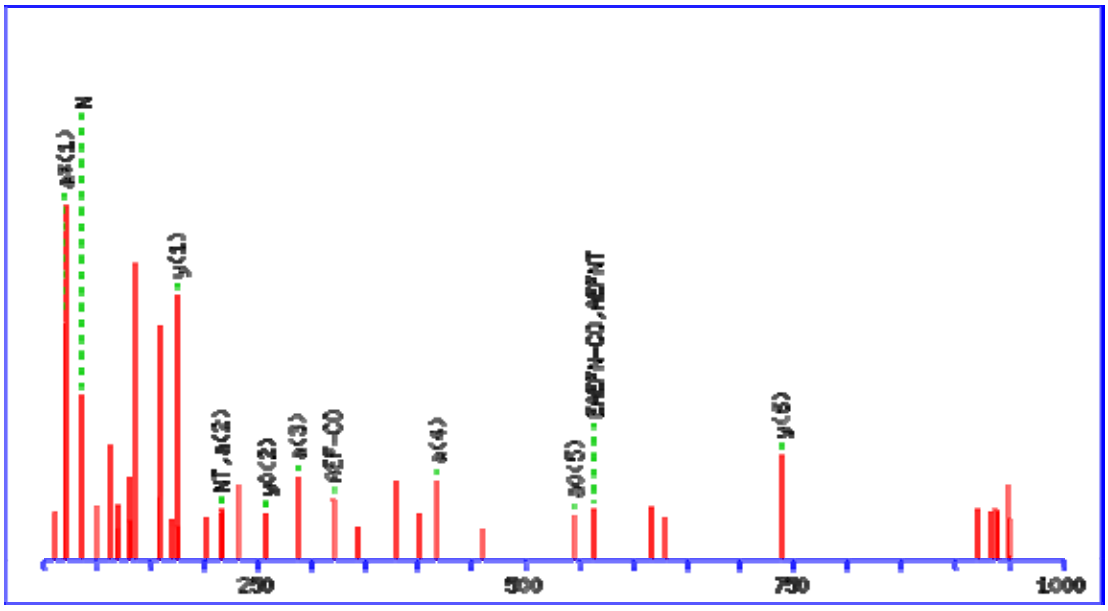


Figure 4.11 Representative CID generated MS/MS spectrum of the amino acid peptide sequence

Amino acid peptide sequence of **DYAEVGR** [$(M+H)^+$ m/z 809.37] and **GSFPWQAK** [$(M+H)^+$ m/z 920.48] from haptoglobin are shown. Only b-, y- and a-type fragment ions are produced that can be used to confirm the amino acid assignments.

Table 4.1 Proteins identified using 2-DE with MALDI TOF-TOF/MS and database search in cyst fluid from EOC

Spot No.	Protein description	Accession Number	No. of peptides sequenced	MOWSE score	Protein coverage (%)	Theoretical MW/p*I*
1	Ceruloplasmin	gi|1620909	3	188	12	116.197/5.43
2	Ceruloplasmin	gi|1620909	7	415	28	116.197/5.43
3	Alpha1-Antitrypsin	gi|1942629	4	270	46	44.280/5.37
4	Chain A, Human Serum Albumin	gi|4389275	3	190	19	68.425/5.67
5	Vitamin D-Binding Protein	gi|21730555	3	89	21	56.34/5.87
6	Chain A, Human Serum Albumin	gi|4389275	4	231	23	68.425/5.67
7	Serum albumin precursor	gi|6013427	8	536	33	71.176/5.91
8	Transferrin	gi|4557871	9	959	40	79.310/6.68
9	HP protein	gi|47124562	7	516	45	31.647/8.48
10	HP protein	gi|47124562	4	229	38	31.647/8.48
11	HP protein	gi|47124562	5	213	56	31.647/8.48
12	IgG kappa chain	gi|4176418	2	133	47	23.726/7.85
13	Proapo-A-I protein	gi|178777	7	527	65	30.745/5.55
14	Hp2-alpha	gi|296653	4	210	21	15.945/5.57
15	Apolipoprotein	gi|27065339	5	190	41	11.291/6.58
16	Transthyretin	gi|55669576	3	191	60	12.835/5.33

4.4 Validation of differentially expressed proteins in cyst fluid using conventional immunologic methods and RT-PCR

4.4.1 Validation of protein identification using 2-DE followed by Western blotting analysis

Aim. To validate ceruloplasmin and haptoglobin identified from 2-DE and MALDI TOF-TOF/MS techniques

Investigation. Cyst fluid from malignant ovarian tumours was selected in this investigation. 2-DE and Western blotting analyses were performed as described previously (section 2.2.10). Rabbit anti-human ceruloplasmin and haptoglobin polyclonal antibodies (Dakocytomation) were used in this validation experiment (section 2.1.2.1). Protein spots of interest were identified according to p*I* and molecular weight of proteins in the parent 2-DE gel using PDQuest software.

Results and interpretation. Ceruloplasmin and haptoglobin identified using MS was confirmed by 2-DE and Western blotting analysis. The polyclonal antibodies used in this technique enabled detection of the corresponding protein spots at the position where they were excised from 2-DE for subsequent identification using MALDI TOF-TOF/MS (Figure 4.12). Validation using the 2-DE followed by Western blotting analysis therefore provides further evidence for the proteomics based identification of protein biomarkers.

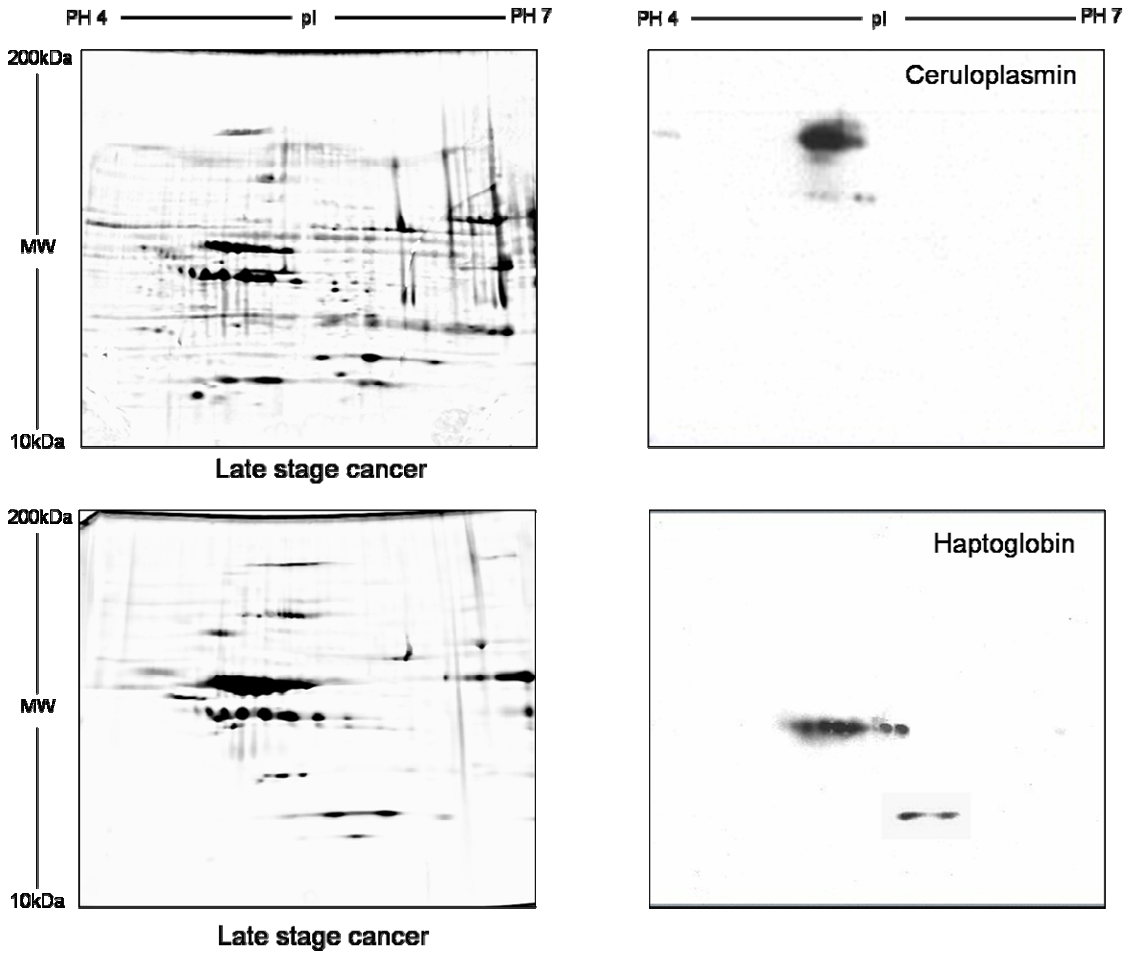


Figure 4.12 Confirmation of identification of ceruloplasmin and haptoglobin using 2-DE followed by Western blotting analysis

Representative cyst fluid 2-DE protein profiles using silver stain (left) and Western blotting analysis of ceruloplasmin and haptoglobin (right) from serous ovarian cancer using 4-20% precast gel. Comparing relative position of protein spots in 2-DE and Western blotting using PDQuest software could confirm the identity of these polypeptides.

4.4.2 Validation of differentially expressed proteins in cyst fluid from benign and malignant ovarian tumours using SDS-PAGE and Western blotting analysis

Aim. To validate differentially expressed ceruloplasmin and haptoglobin in cyst fluid using SDS-PAGE and Western blotting methods

Investigation. Cyst fluid proteins from benign and malignant ovarian tumours were subjected to 15% SDS-PAGE as described earlier (section 2.2.5). Polyclonal rabbit anti-human antibodies were used for Western blotting analysis (section 2.2.10). Ceruloplasmin was analysed using native gel electrophoresis without application of DTT and SDS, and high temperature was also avoided to prevent fragmentation of this high molecular weight protein (section 2.2.6). Band intensities from Western blotting were measured using Quantity One® software (Bio-Rad Laboratories)

Results and interpretation. The antibody used in this experiment could detect the different subunits of ceruloplasmin and haptoglobin. For ceruloplasmin, previous studies indicated that this protein may undergo proteolytic fragmentation and result in segments with different molecular weight (Kingston et al., 1977). Hence, in this experiment, ceruloplasmin was analysed by native gel electrophoresis in order to facilitate the comparison of intensity difference between the benign and malignant ovarian tumours. It was observed that ceruloplasmin antibody produced a major band corresponding to the molecular weight of 130 kDa (Figure 4.13). On the other hand, the haptoglobin antibody revealed three subunits of this acute phase reactant with molecular weights of 40 kDa, 17.5 kDa and 9 kDa as mentioned previously (section 3.5.1; Figure 4.13). Moreover, the intensities of both proteins in cyst fluid were significantly higher in late stage ovarian cancer when compared with benign ovarian tumours (Figure 4.13, $P<0.01$).

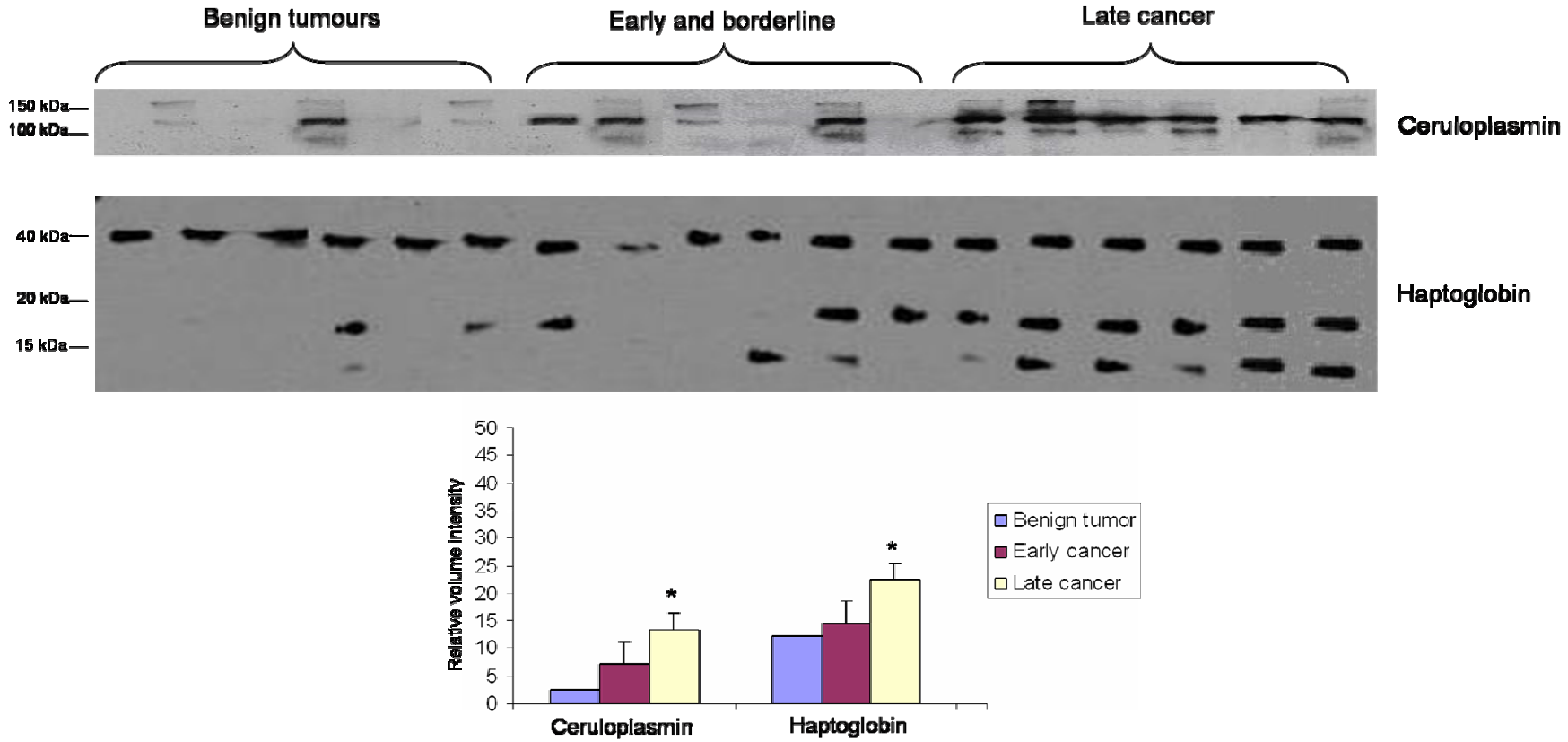


Figure 4.13 Western blotting analysis of ceruloplasmin and haptoglobin in cyst fluid from benign, borderline tumour, early and late stage ovarian cancers

The intensities of both proteins were significantly elevated in cyst fluid from late stage ovarian cancer when compared with benign tumours (*P<0.01).

4.4.3 Validation of differentially expressed proteins in tissues from benign and malignant ovarian tumours using Western blotting analysis

Aim. To explore the aberrant expression of ceruloplasmin and haptoglobin in the ovarian tumour tissues

Investigation. Haematoxylin and eosin (H & E) staining was conducted to visualise sections containing the malignant ovarian cancer cells (section 2.2.16). For Western blotting analysis, the tissue sections were carefully selected following consultation with the histopathologist wherein the cancer cells accounted for the major portion of the tissue. The samples used were placed in lysis buffer (section 2.1.2.3) followed by homogenisation. Following centrifugation, the supernatants were collected and used for the subsequent experiments.

Results and interpretation. H & E staining from representative serous and mucinous ovarian tumours is indicated in Figure 4.14. It was observed that cancer cells were localised to form “cancer loci” with high nucleus to cytoplasm ratio. The neoplastic cells had large and irregular nuclei compared with para-cancerous interstitial cells and those from benign ovarian tumours. Samples from early stage ovarian cancer were not included in this study because of paucity of material. Our data revealed that significant up-regulation of ceruloplasmin ($P<0.01$) and haptoglobin ($P<0.01$) could be observed in ovarian cancer tissues when compared with those from benign tumours (Figure 4.15). Moreover, as indicated in Figure 4.16, a significant positive correlation existed between the levels of these two proteins in cyst fluid and in the corresponding ovarian tumour tissues. The positive correlation between tissue and cyst fluid levels of ceruloplasmin and haptoglobin therefore provides evidence that alteration of both

proteins in cyst fluid may represent changes in the microenvironment of ovarian tumours.

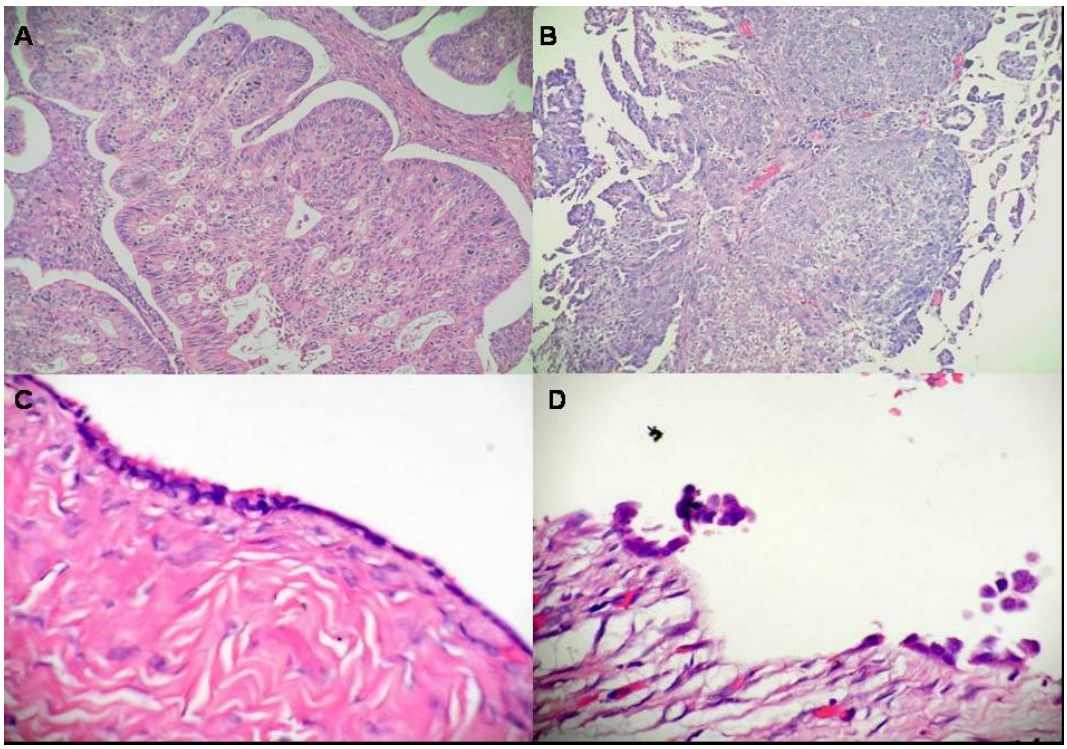
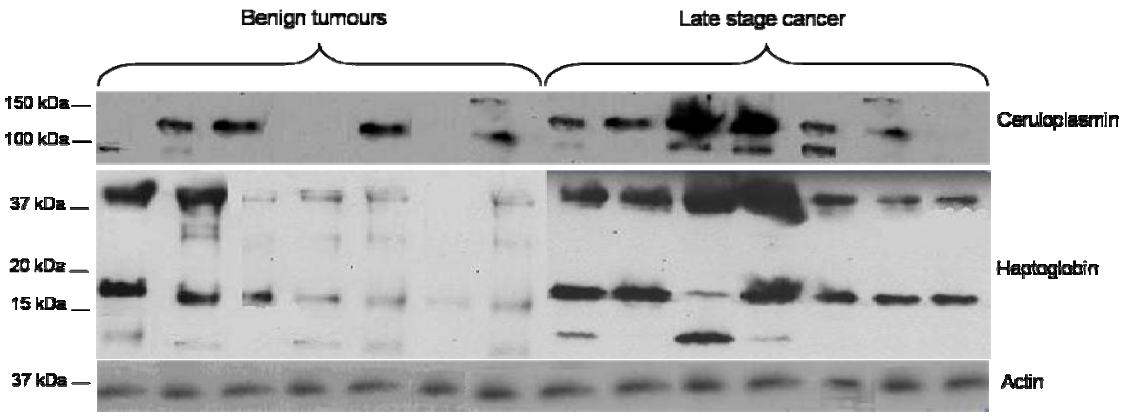


Figure 4.14 H & E staining of representative benign and malignant ovarian tumours

A serous cancer; B mucinous cancer; C benign serous tumour; D benign mucinous tumour. For tissue selection, the neoplastic cells accounted for the major portion of the tissue sections. As indicated, the malignant cells possessed the typical characteristics of large and irregular nuclei compared with those from benign ovarian tumours. Original magnification 200X

A



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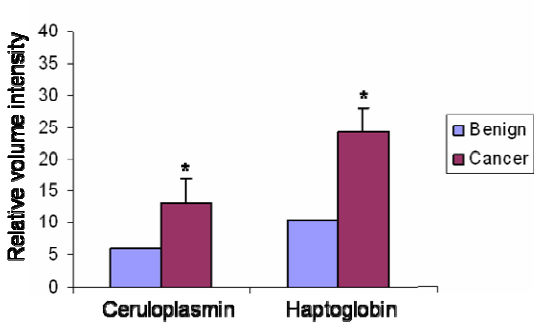


Figure 4.15 Differential expressions of ceruloplasmin and haptoglobin in benign and malignant ovarian tumour tissues

A Differentially expressed ceruloplasmin and haptoglobin in tissues from benign and malignant ovarian tumours detected by Western blotting analysis. **B** The expression of ceruloplasmin and haptoglobin was significantly higher in ovarian cancer tissue compared with those from benign ovarian tumours. The bars represent the mean ± SEM of densities of gel bands determined from ovarian cancer patients and controls. (* $P<0.01$)

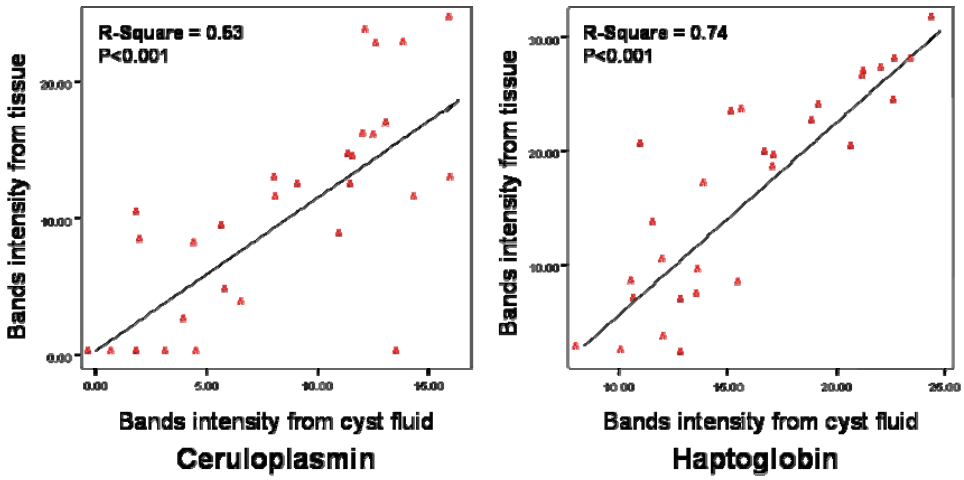


Figure 4.16 Correlations of haptoglobin and ceruloplasmin expression in cyst fluid and tissue

Single regression analysis of band intensities in ovarian tumour tissues and cyst fluids is demostrated. Significant correlations were observed between levels of the proteins in cyst fluid and the corresponding tissue samples (P<0.001).

4.4.4 Immunohistochemical localisation of ceruloplasmin and haptoglobin in benign and malignant ovarian tumours

Aim. To localise the presence of cerulopasmin and haptoglobin in ovarian tumour tissue sections by the immunohistochemical technique

Investigation. Immunohistochemical experiments were carried out in wax-embedded and frozen tissue sections. Samples were embedded in wax or subjected to frozen section after collection and H & E staining was performed. Sections used were carefully selected after consultation with the histopathologist. Polyclonal rabbit anti-human ceruloplasmin and haptoglobin antibodies were used in this study (section 2.1.2.1).

Results and interpretation. In accordance with results from the Western blotting procedure, immunohistochemical analysis also indicated intense staining of ceruloplasmin and haptoglobin in the cytoplasm of ovarian cancer cells obtained from late stage ovarian cancers when compared with those from benign ovarian tumours (Figure 4.17). Cellular staining was mostly cytoplasmic with the majority of the staining being observed in “cancer loci”. Strong staining for haptoglobin was evident in areas with stroma and vascular spaces as well as in the ovarian vessels. Coupled with the Western blotting data in section 4.4.2 and 4.4.3, it is reasonable to suggest that levels of ceruloplasmin and haptoglobin were up-regulated in both cyst fluid and tissue levels from the malignant tumours. Elevation in these two proteins in cyst fluids and tissues could be indicative of the biosynthetic ability of ovarian cancer cells.

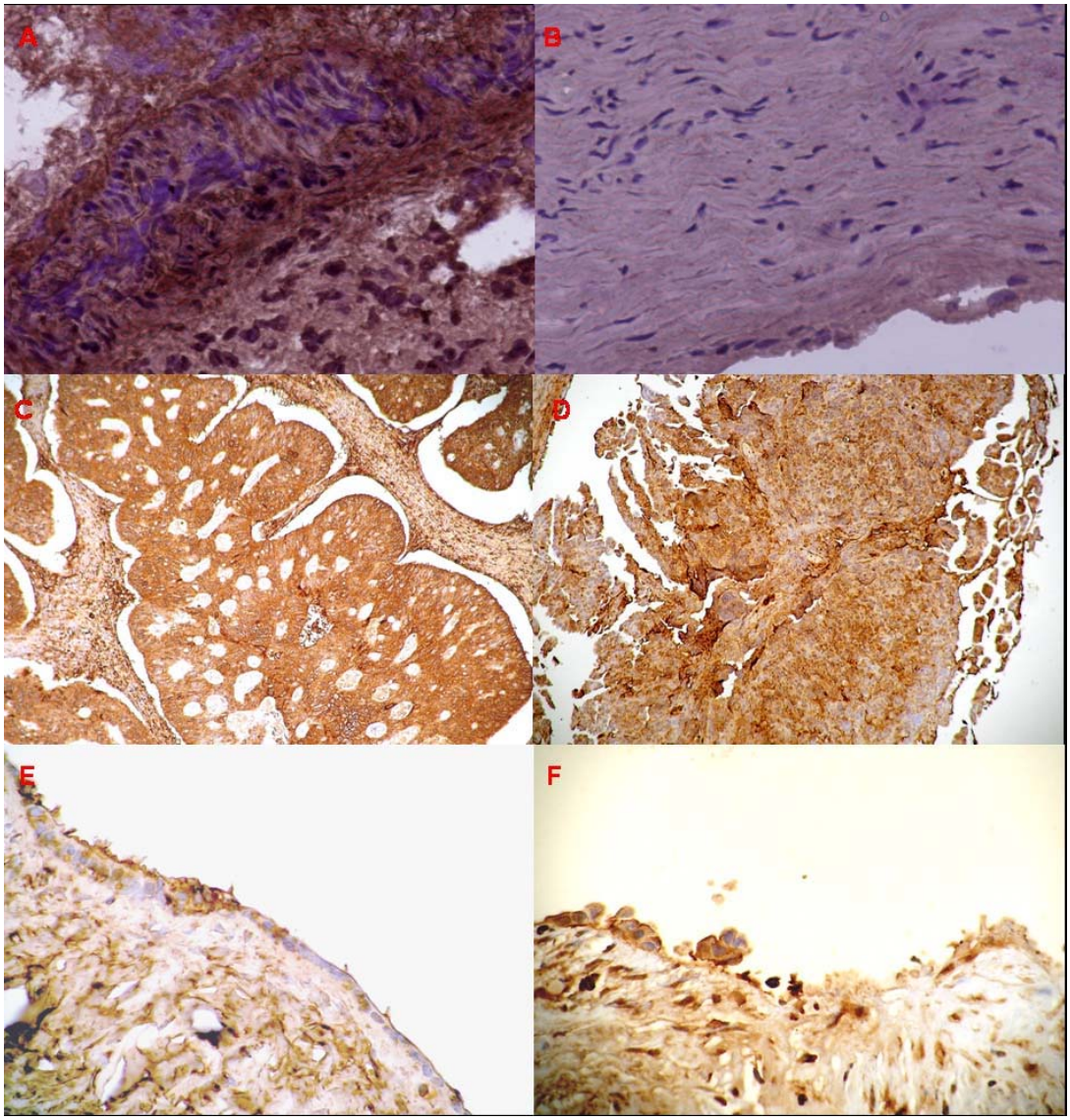


Figure 4.17 Differential expressions of ceruloplasmin and haptoglobin in ovarian tumour tissue samples examined by immunohistochemistry. Ceruloplasmin was expressed predominantly in late stage ovarian cancer (A) compared with benign ovarian tumours (B). Haptoglobin was expressed in both late stage ovarian cancer (C serous, D mucinous) and benign ovarian tumour (E serous, F mucinous). The expression level was higher in ovarian cancer cells when compared with those from benign tumours. More intense staining was evident in areas with cancer cells as well as in the vascular spaces. Original magnification ×200

4.4.5 Quantitative measurements of ceruloplasmin and haptoglobin in cyst fluid from benign and malignant ovarian tumours using in-house ELISA method

Aim. Using Western blotting and immunohistochemistry studies we have evaluated the relative expression levels of ceruloplasmin and haptoglobin in benign and malignant cyst fluid as well as in tumour tissues. The in-house ELISA methods were employed to quantify these two proteins in cyst fluid.

Investigation. Cyst fluid samples from 47 benign ovarian tumours, 13 borderline tumours, 10 early stage and 20 late stage ovarian cancers were used in this study. For ceruloplasmin, the 96-well microplates were coated with cyst fluid antigen directly and a rabbit anti-human ceruloplasmin antibody (Dakocytomation) was used to detect its concentration. For haptoglobin, instead, a sandwich ELISA method was developed using two antibodies specific to haptoglobin (section 2.2.12). Briefly, cyst fluid samples were diluted 100 times for haptoglobin and undiluted samples were used for ceruloplasmin measurements. Purified ceruloplasmin and haptoglobin (Sigma grade) were used as standards. Standard curves were obtained by serial dilution of the corresponding purified proteins.

Results and interpretation. Tetrameric protein haptoglobin has three phenotypes Hp 1-1, 2-1, and 2-2 (Tseng et al., 2004a). All the phenotypes are composed of different combinations of β, α_2 and α_1 subunits. Results from western blotting analysis showed that the two antibodies used for the in-house sandwich ELISA method possessed the capacity of binding both alpha and beta subunits (Figure 3.7 and 4.18 and 4.12). Based on this finding, the ELISA method used in this study measured the total serum haptoglobin concentrations. The intra- and inter-assay CVs were computed by using three samples of different concentrations 8 times on the same day and on 5 consecutive days, respectively. The CVs were

4.8% and 15.3% for the intra-assay and inter-assays for ceruloplasmin measurements while it was 3.8% and 14.2% for haptoglobin, respectively. A representative standard curve for haptoglobin is given in Figure 4.19. Comparison of the mean of haptoglobin or ceruloplasmin levels in benign subjects and those presenting with early and late stage cancer were analysed using the ANOVA analysis. The concentration of haptoglobin in cyst fluid from early (4.44±0.61 mg/ml) and late stage (5.96±0.56 mg/ml) ovarian cancer was significant higher when compared with benign ovarian tumours (1.54±0.30 mg/ml, $P<0.01$). No significant difference in ceruloplasmin concentrations was discernible between early, late stage ovarian cancers (0.89±0.36; 1.11±0.18 µg/ml respectively) and benign ovarian tumours (0.65±0.11 µg/ml; Table 4.2 and Figure 4.20).

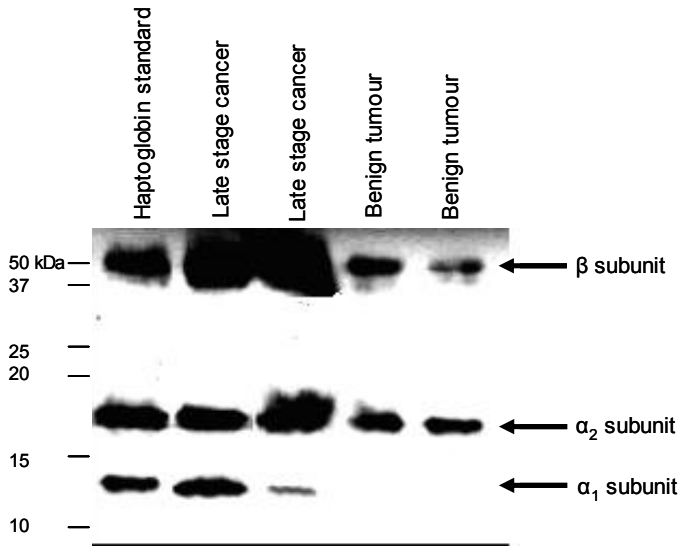


Figure 4.18 Western blotting analysis showing binding capacity of goat anti-human haptoglobin antibody used in ELISA

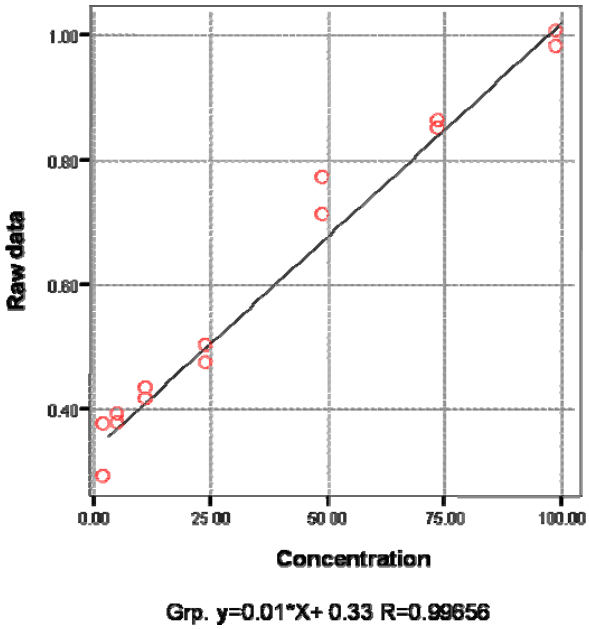


Figure 4.19 Representative standard curve obtained from a serial dilution of purified haptoglobin.

Table 4.2 Mean concentrations of ceruloplasmin and haptoglobin in cyst fluids from benign and malignant ovarian tumours by the ELISA method

Group	Haptoglobin level			Ceruloplasmin level	
	No.	Mean (mg/ml)	P	Mean (μg/ml)	P
Late cancer	20	5.96±0.56	<0.01	1.11±0.18	=0.134
Early and borderline	23	4.44±0.61	<0.01	0.89±0.36	
Benign tumour	47	1.54±0.30		0.65±0.11	

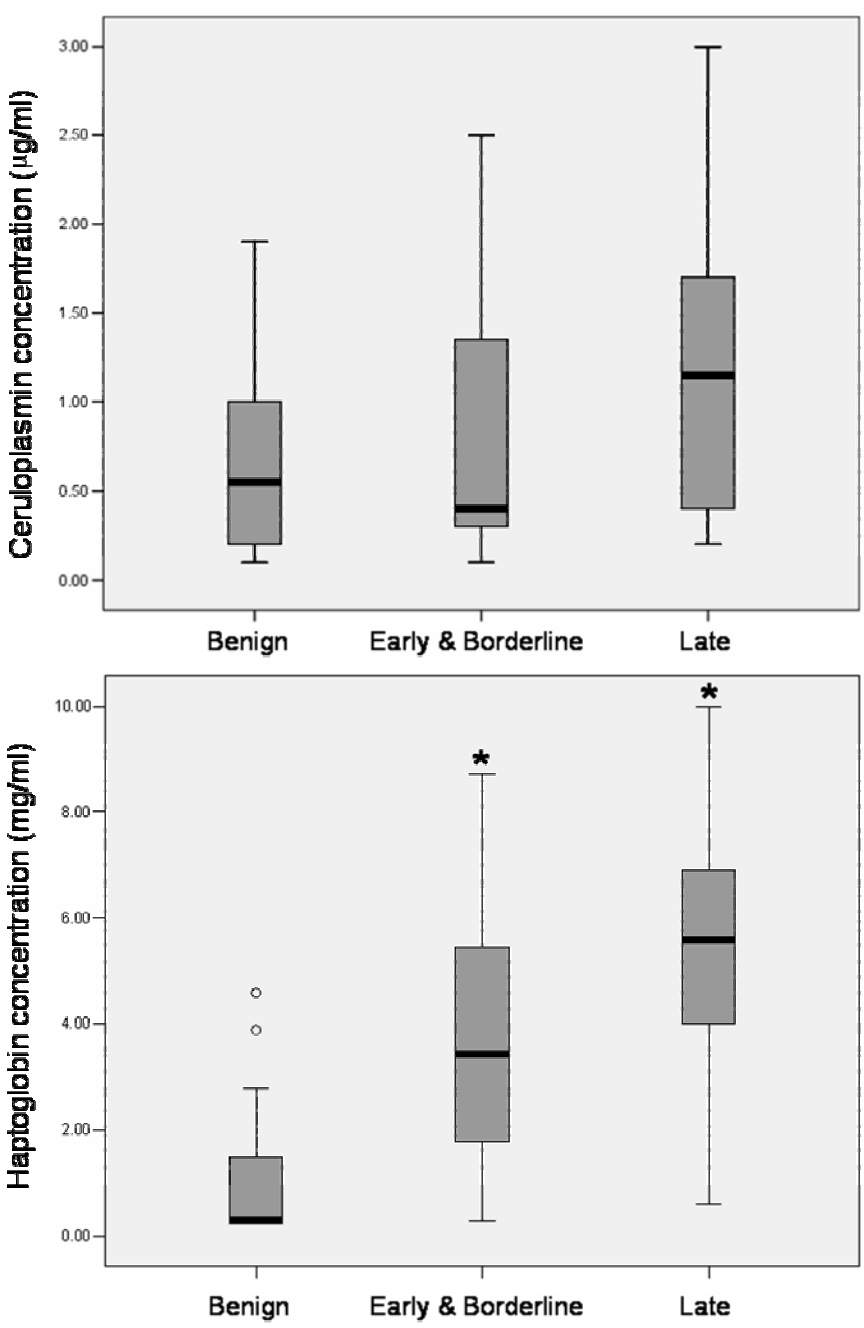


Figure 4.20 Concentration distribution of cyst fluid ceruloplasmin and haptoglobin

Cyst fluid ceruloplasmin and haptoglobin concentrations obtained from 20 patients with EOC, 23 with early and borderline and in 47 benign ovarian tumours. The haptoglobin levels were significantly higher in patients with early and late stage ovarian cancers when compared with those from benign tumours (*P<0.01). There was no significant difference between early, late stage ovarian cancer and benign ovarian tumours for ceruloplasmin (P=0.134).

4.4.6 Validation of ceruloplasmin and haptoglobin mRNA expression in ovarian cancer cell lines using RT-PCR

Aim. Expression of haptolgobin and ceruloplasmin proteins were evaluated in cyst fluid and tissue samples from benign and malignant ovarian tumour patients. The RT-PCR was conducted to examine the mRNA expression of these biomolecules in three ovarian cancer cell lines.

Investigation. Three ovarian cancer cell lines were obtained from ATCC and cultured until a density of about 80% confluence. Total RNA was extracted (section 2.2.2 and section 2.2.17) from approximately $5X10^6$ cells followed by quantitative and qualitative measurements (section 2.2.17). Optimal PCR conditions for each gene was determined in preliminary experiments so that the number of cycles selected was within the linear range of amplification and optimal annealing temperatures were determined for each gene (Agarwal et al., 2001). Primer pairs used for the amplification of the individual gene are listed in Table 4.3. The conditions for RT-PCR reaction in this experiment is illustrated in Table 4.4.

Table 4.3 Primer pairs used for the amplification for individual gene

Target proteins	Sense	Anti-Sense	Amplified bps
Haptoglobin	5'-CTG TGC TGG CAT GTC TAAG-3'	5'-CAG CTA TGG TCT TCT GAAC-3'	197
Ceruloplasmin	5'-GGCCCAGAACCCTGGAGAATGG-3'	5'-CTGCTCTGGGGGTTGTAATTTGG-3'	518
GAPDH	5'-AAGGACTCATGACCACAGTCCATG-3'	5'-TTGATGGTACATGACAAGGTGCGG-3'	673

Results and interpretation. Denaturing formaldehyde agarose gel electrophoresis revealed the presence of 18S rRNA and 28S rRNA bands, which indicated the integrity of total RNA in these experiments (Figure 4.21). An intense mRNA expression for haptoglobin occurred in the three ovarian cancer cell lines (Figure

4.22), which provided confirmatory evidence that the tumour cells might possess the capacity to synthesise haptoglobin. However, ceruloplasmin mRNA expression was not observed in these cell lines.

Table 4.4 RT-PCR reaction condition

Step	Time	Temperature (^{o}C)
Reverse transcription:	30 min	50
Initial PCR activation step	15 min	95
Three-step cycling (35-40 cycles)		
Denaturation	45 sec	94
Annealing	45 sec	variable
Extension	1 min	72
Final extension	10 min	72

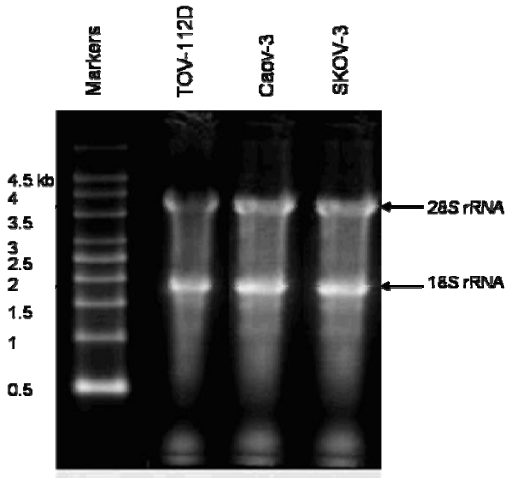


Figure 4.21 Analysis of integrity of total RNA from ovarian cancer cells using denaturing formaldehyde agarose gel electrophoresis

Presence of 28S rRNA and 18S rRNA demonstrated the integrity of RNA

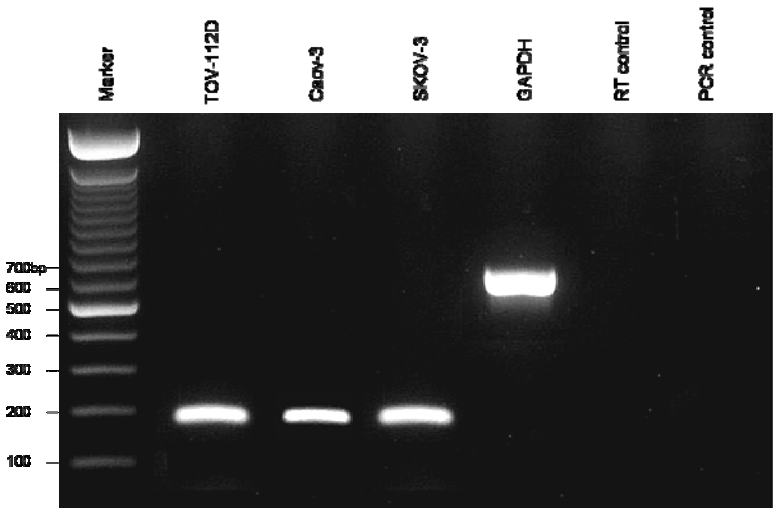


Figure 4.22 Analysis of mRNA expression of haptoglobin in ovarian cancer cell lines by RT–PCR

A fragment of 197 base pairs was amplified from the total RNA isolated from ovarian cancer cell lines. RT control was conducted without Sensiscript reverse transcriptase. PCR control was conducted without RNA template.

4.5 Conclusions

The progression from early to late malignant ovarian cancer is accompanied by genetic alterations and changes in protein levels which are of critical importance since altered protein expression is a hallmark of neoplasia. Therefore, proteins secreted by ovarian cancer cells into cyst fluid may prove to be useful markers for intra-operative discrimination of malignant from benign ovarian tumours. They may also provide a rich source of potential tumour markers which are more difficult to be isolated and identified in blood where there are a number of abundant proteins.

In this regard, the discovery of sensitive and specific serum biomarkers for the diagnosis and prognosis of ovarian cancer remains a daunting challenge. The large dynamic range between the least and most abundant of protein constituents found in biological fluids is a difficult hurdle to overcome. The current study approaches this problem by removing the most abundant circulating protein, albumin from cyst fluid samples which make up more than 70% of total serum protein by mass. The immunoaffinity-depleted cyst fluid samples from patients with ovarian cancer and benign samples were then subjected to 2-DE. 2-DE coupled with MS is a key separation technique in proteome analysis due to its advantage of simultaneous separation and display of many proteins at a time with good reproducibility (Gorg et al., 2004), while MS is a powerful tool for mining with high sensitivity and speed of analysis for the 2-DE proteome. This study demonstrated for the first time the presence of differentially expressed ceruloplasmin and haptoglobin in cyst fluid from ovarian cancers when compared with benign ovarian tumours.

Another interesting observation is that relative quantitative Western blotting using SDS-PAGE demonstrated significant higher intensities of ceruloplasmin and haptoglobin in cyst fluid and tissues from patients with ovarian cancer. Localisation of ceruloplasmin and haptoglobin by immunohistochemistry also indicated enhanced staining of both proteins in ovarian cancer cells from the tissue sections. Moreover, measurements of haptoglobin concentration in cyst fluid using the in-house sandwich ELISA method indicated elevation of haptoglobin in cyst fluid from malignant ovarian tumours. Therefore, it seems plausible to suggest that elevated cyst fluid haptoglobin levels could be derived from the malignant tumour cells, although the circulating molecules may also contribute to this elevation.

In conclusion, using conventional 2-DE based proteomics methods combined with immunological validation procedures, this study indicates the differential expression of ceruloplasmin and haptoglobin in cyst fluid from benign and malignant ovarian tumours. Identification of high molecular weight proteins including ceruloplasmin and haptoglboin-β subunit using 2-DE indicates that this high throughput technology could serve as a complementary method to SELDI-TOF in identifying candidate protein markers for EOC. It is noteworthy that haptoglobin may be a useful marker for diagnosis and prognostication of malignancy in women presenting with this insidious disease.

Chapter 5: Investigations on haptoglobin as a diagnostic and prognostic marker for EOC

5.1 Introduction

Significant differences in survival rates between early and advanced EOCs has been the focus of research to identify markers for EOC that can be of diagnostic or prognostic value. Owing to the paucity of symptoms and their insidious onset, most ovarian cancer patients present with advanced stage of the disease, the five-year survival rate being a mere 30% often due to a failure in early diagnosis (Menon, 2004). Although CA-125 is by far the most useful biomarker for ovarian cancer, it is only limited in evaluations of therapeutic effectiveness in patients diagnosed with EOC and also in detection of recurrent disease during post-treatment surveillance (Meyer et al., 2000). Moreover, CA-125 is not a specific biomarker for ovarian cancer due to its elevation in women with other benign gynaecologic conditions such as ovarian cysts, endometriosis and uterine fibroids (Mogensen, 1992; Cannistra, 1993; Mackey et al., 1995). On the other hand, prognosis of ovarian cancer is largely dependent on the stage and histologic grade on presentation. Patients with well-differentiated tumours based on histologic grade tend to have a better survival rate compared to those with poorly-differentiated tumours (Swenerton et al., 1985). The current management of patients with ovarian cancer involves optimal cytoreductive surgery followed by chemotherapy. Despite an initial favourable reaction to the therapy, most patients however, appear to have a relapse within five years after management of the tumours (Ozols, 2005). The present study therefore focuses on the

evaluation of clinical significance of haptoglobin as a biomarker identified from the cyst fluid of EOC.

Data from this research revealed that haptoglobin was differentially expressed in samples from benign and malignant ovarian tumours. The applicability of this biomolecule needs to be further evaluated in our group of patients attending the Gynaecologic Oncology Clinic at the National University of Hospital, Singapore. Two important clinical evaluations were conducted to address this issue. Firstly, the hypothesis 2 “Similar protein expression patterns may also observed in serum from benign and malignant ovarian tumours and they may have potentials to be diagnostic and prognostic markers” was examined by measurements of haptoglobin concentration in sera from patients with EOCs. Secondly, we explored the hypothesis 3 in which “an intra-operative assay using cyst fluid could be developed so as to differentiate malignant from benign ovarian tumours at the time of surgery”. In this regard, a simple, portable and rapid intra-operative diagnostic kit which could diagnose malignant ovarian tumours in the operation was developed.

5.2 Pre-operative diagnostic and prognostic significance of haptoglobin in sera of patients with epithelial ovarian tumours

Aim. The development of non-invasive diagnostic and prognostic strategies for ovarian cancer detection has been the Holy Grail for several decades. The commonly employed techniques involve serum marker measurement using ELISA which is a reliable, sensitive and widely acceptable method for the

identification and monitoring of cancer. In this study, the clinical significance of serum haptoglobin levels was evaluated in EOC.

Investigation. Blood samples from benign and malignant ovarian tumours were collected during surgery without intra-operative spillage (section 2.2.1.2). The total haptoglobin levels were quantified using the sandwich ELISA technique with two polyclonal antibodies against this protein (section 2.2.12). The diagnostic potential of serum haptoglobin was evaluated by the ROC curve analysis and compared to the conventional biomarker CA-125. This analysis represents the standard procedure in evaluating the performance of putative diagnostic biomarkers (Shapiro, 1999).

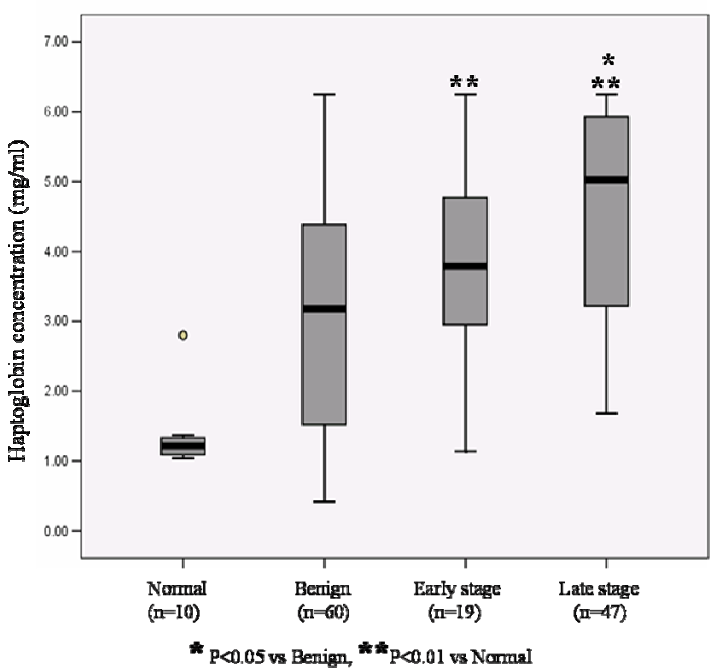
Moreover, the overall cancer-specific survival was defined to be from the date of blood sampling to the date of death due to malignancy. Univariate and multivariate analyses were performed to estimate the probability of survival and the differences in survival rates. In order to evaluate the acute phase reactions in the patients with EOC, serum concentrations of CRP were also determined by specific ELISA kits (Chemicon International) in accordance with the manufacturer's instructions. In addition, thirteen EOC patients who were treated with combination therapy consisting of carboplatin (AUC 5) and taxol (175 mg/m^2 body weight) following surgery were included in this study. Blood samples were taken prior to surgery and after six cycles of chemotherapy with a view to assess serum haptoglobin concentrations in evaluating therapeutic effectiveness for these patients.

Results. Data from this study indicated that serum haptoglobin concentrations were significantly higher in late stage ovarian cancer patients (4.6±0.6 mg/ml) when compared with benign ovarian tumour and healthy controls (3.12±0.5

mg/ml, 1.5±0.5 mg/ml respectively; P<0.05, P<0.01). Moreover, a significant difference was observed in haptoglobin levels between early stage cancer (3.6±0.5 mg/ml) when compared with healthy controls (P<0.01, Figure 5.1). This observation appears to be in agreement with previous reports (Warwas et al., 1981; Dobryszycka et al., 1999).

Since CRP is a serological marker that can be used to investigate the association between inflammation and risk of cancer, the levels of this acute phase reactant were measured in our patients. CRP concentrations were observed to be significantly elevated in both early and late stage EOCs (13.9±3.0µg/ml; 12.7±2.7µg/ml respectively) when compared with normals as well as benign tumours (1.28±0.4µg/ml; 2.67±0.9µg/ml, P<0.01). The distribution of haptoglobin and CRP levels in benign, early and late EOC as well as in normal women is presented in the boxplot which is bounded by the 75^{th} and 25^{th} percentiles (Figure 5.1). Moreover, a significant elevation in serum haptoglobin and CRP levels was observed in early and late stage EOC when compared with those from normals and benign tumours as illustrated in Figure 5.1.

The diagnostic potential of serum haptoglobin was evaluated using ROC analysis and compared to the conventional ovarian cancer marker CA-125. Area under the curve (AUC) was computed to test the inherent capacity of a biomarker for discriminating between diseased and non-diseased subjects (Hanley et al., 1982). Using this parameter, it was observed that the AUC for haptoglobin and CA-125 was 0.741 and 0.758 respectively (Figure 5.2). The relatively low AUC for haptoglobin therefore limited its diagnostic power in the clinical settings.



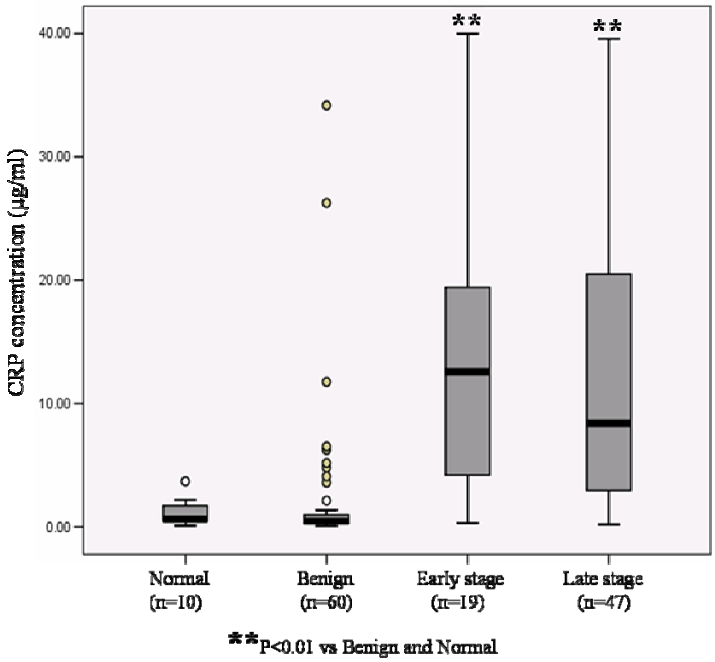
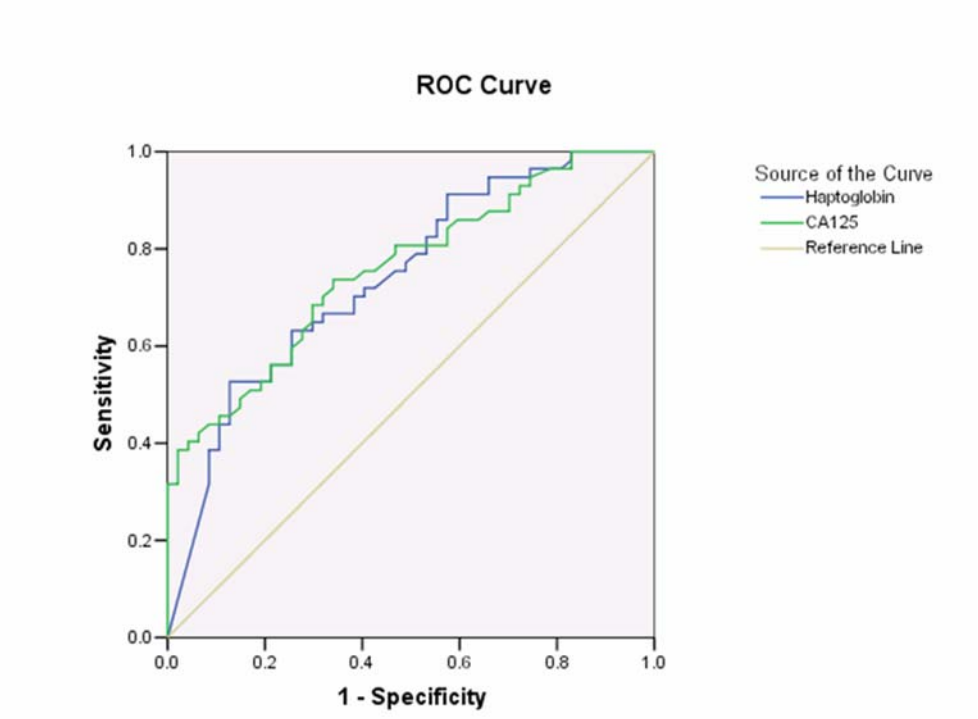


Figure 5.1 Concentration distribution of serum haptoglobin and CRP

Serum haptoglobin and CRP concentrations obtained from 66 patients with EOC, 60 benign ovarian tumours and 10 healthy controls. Serum levels of haptoglobin and CRP were significantly higher in patients with early and/or late stage of ovarian cancer than in control subjects.



Area Under the Curve

Test Result Variable(s)	Area
Haptoglobin	.741
CA125	.758

Figure 5.2 Evaluation of diagnostic potential of haptoglobin using ROC curve analysis

The diagnostic potential of serum haptoglobin from epithelial ovarian tumours was evaluated using ROC curve analysis and compared to the conventional marker CA-125. The AUC for these two biomarkers are 0.741 and 0.758 respectively.

The association of haptoglobin levels with clinicopathologic variables including age, FIGO stage, tumour type, Grade and pathology is given in Table 5.1. In this study, we have used a cut-off value of 4 mg/ml of concentration for haptoglobin since this value corresponds to a better diagnostic accuracy in our series of patients. A cut-off value of 4 mg/ml minimises the false positive and negative rates in the differentiation of normal and benign cases from the malignant ovarian tumours. As indicated in the ROC curve, the largest value of the sum of sensitivity and specificity could be achieved by adding a series of sensitivity and specificity at different cut off coordinate points at 4.0mg/ml (Figure 5.3). Accordingly, following this criteria, the ovarian cancer patients were divided into two groups based on haptoglobin concentrations. The tumour staging/grading according to the International Federation of Gynecology and Obstetrics (FIGO) classification is known to be the most important prognostic factor for ovarian cancer. In this study, we noted a significant association between the serum haptoglobin level and FIGO stage at diagnosis ($P<0.05$, Table 5.1). However, no significant association was observed between the serum haptoglobin level and age, histology, or tumour grade at diagnosis ($P>0.05$).

In addition, tumour haptoglobin expression levels were also examined for the overall survival rates using the Kaplan-Meier analysis with log-rank statistics for determining significance. The mean follow-up time for the entire cohort of 66 patients was 37.7 months (range, 4-100 months). Using the 4 mg/ml haptoglobin concentration as a cut-off point mentioned earlier, the median and mean survival time of patients with enhanced serum haptoglobin levels were observed to be significantly less when compared to those with low serum levels for this acute phase reactant protein (38 versus 79, and 46.7 versus 84.7 months; $P = 0.048$). Kaplan-Meier survival curves generated for high versus low concentrations of haptoglobin are illustrated in Figure 5.4. It is noteworthy that significantly

elevated haptoglobin concentrations were associated with poor outcome for survival in our women with EOC (Figure 5.4, P = 0.048).

Using the Cox proportional hazards model, multivariate survival analysis of 66 patients we observed enhanced haptoglobin levels (relative risk, 5.054; 95% confidence interval, 1.11-22.98) and tumour stage at diagnosis (relative risk, 5.99; 95% confidence interval, 1.24-29.0) which appeared to be significant predictor variables of overall survival in these patients. In contrast however, age, tumour type, histology and grade at diagnosis were not significantly correlated with survival rates as illustrated in Table 5.2.

Haptoglobin levels in sera from 13 patients who were subjected to chemotherapy were also measured in order to determine whether chemotherapeutic treatment had any effect on the expression of this acute phase reactant protein. Its concentration was significantly reduced after six cycles of chemotherapeutic treatment (2.2 ±0.8 mg/ml) when compared with those before treatment (5.2±0.7 mg/ml; P<0.001). This result is in accordance with a previous study (Ahmed et al., 2005), indicating a role for haptoglobin in monitoring patients undergoing chemotherapy.

Interpretation. It is envisaged that inflammation could serve as a potential contributory factor in the development of a variety of cancers. CRP is a serological marker of inflammation that could be utilised to investigate the association between inflammation and risk of cancer (Helzlsouer et al., 2006). It is noteworthy that in a similar manner to that of haptoglobin, serum CRP levels were also significantly elevated in early and late stage ovarian cancer. Moreover, a significant correlation existed between CRP and haptoglobin levels (r^2=0.22, P<0.001; Figure 5.5) indicating that inflammation occurs in the early and late

stages of ovarian cancer development. Based on this observation, it seems plausible to suggest that CRP could serve as an adjunct to haptoglobin in the evaluation of ovarian cancers. Since haptoglobin is among the most abundant glycoprotein secreted by the liver and circulating hepatic haptoglobin accounts for the major elevation of this protein in the serum of ovarian cancer patients (Ahmed et al., 2005), it is reasonable to suggest that enhanced hepatic synthesis of this protein occurs due to an acute phase response in these ovarian cancer patients.

Haptoglobin levels in sera from 13 patients who were subjected to chemotherapy were also measured in order to determine whether chemotherapeutic treatment had any effect on the expression of this acute phase reactant protein. Its concentration was significantly reduced after six cycles of chemotherapeutic treatment (2.2 ±0.8 mg/ml) when compared with those before treatment (5.2±0.7 mg/ml; $P<0.001$). This result is in accordance with a previous study (Ahmed et al., 2005), indicating a role for haptoglobin in monitoring patients undergoing chemotherapy.

Using the immunohistochemical technique described earlier (section 4.4.4) strong intensity of staining for haptoglobin was observed in the ovarian cancer cells and blood vessels suggesting that other extrahepatic sources may also contribute to its elevation in the blood of EOC patients. Moreover, the enhanced haptoglobin concentrations in cyst fluid may be reflective of its biosynthesis from malignant ovarian tumours. It has been reported that a substantial portion of the synthesised haptoglobin occurs as a single polypeptide precursor (Misumi et al, 1983). It is also noteworthy that circulating levels of this acute phase reactant could be the result of increased intrahepatic synthesis.

Our retrospective analysis therefore provides evidence that circulating haptoglobin levels could be a useful independent prognostic indicator in patients with EOC. Overall, our data suggests that lower haptoglobin levels in pre-operative sera are associated with better survival outcome.

Table 5.1 Association of serum haptoglobin levels with clinicopathologic variables

Variable	n	Haptoglobin concentration		P*
		Low (<4mg/ml)	High (>4mg/ml)	
Age				
<52	31	13	18	0.691
≥52	35	13	22	
Tumour type				
Benign	60	41	19	<0.001
Malignant	66	26	40	
FIGO stage				
Early (I/II)	19	11	8	<0.05
Late (III/IV)	47	15	32	
Histology				
Serous	27	11	16	0.254
Mucinous	20	8	12	
Endometrioid	11	5	6	
Clear cell	6	0	6	
No record	2			
Differentiation				
Well	24	8	16	0.918
Moderate	14	5	9	
Poor	23	9	14	
No record	5			

* The Pearson's χ^2 test was used to compare the demographic and clinical data.

Table 5.2 Multivariate survival analysis by Cox regression

Variables	Hazard ratio (95% CI)	P
Age	1.032 (0.357—2.981)	0.954
FIGO stage	5.99 (1.237—29.005)	0.026
Histology	0.839 (0.522—1.346)	0.466
Grade	1.162 (0.457—3.035)	0.896
Haptoglobin	5.054 (1.111—22.983)	0.036

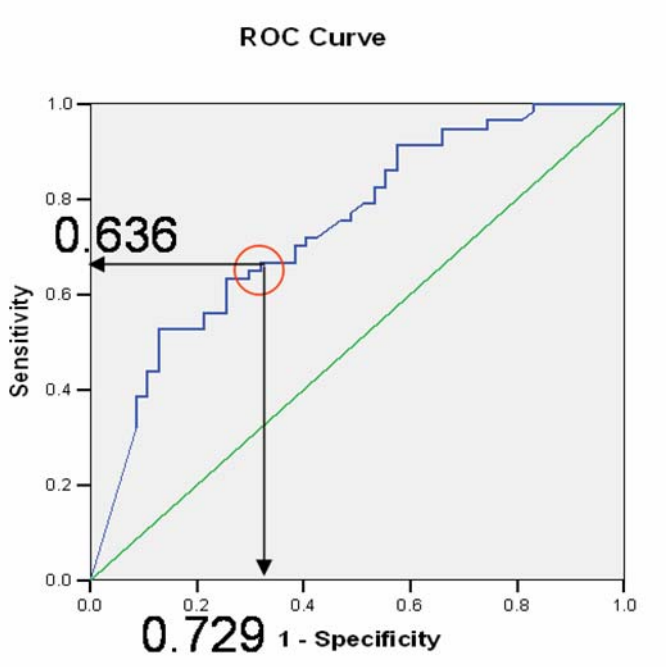


Figure 5.3 Determining suitable serum haptoglobin cut-off value for prognosis analysis using ROC curves analysis
Using different cut off coordinate points a series of sensitivity and specificity would be generated. It was noted that at the point of 4.0mg/ml the greatest value of the sum of sensitivity and specificity could be achieved.

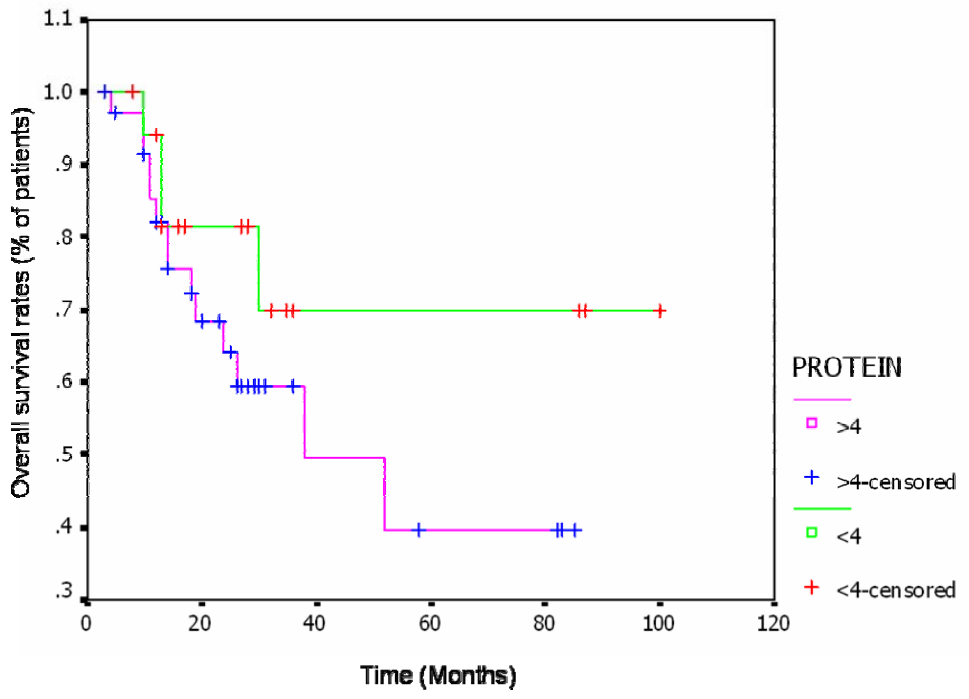


Figure 5.4 Univariate survival analysis of EOCs
Overall survival of 66 patients with EOC in relation to haptoglobin level was indicated. A cut-off value of 4 mg/ml was used to divide the patient into two groups. Patients with higher haptoglobin concentrations had a significantly worse survival probability.

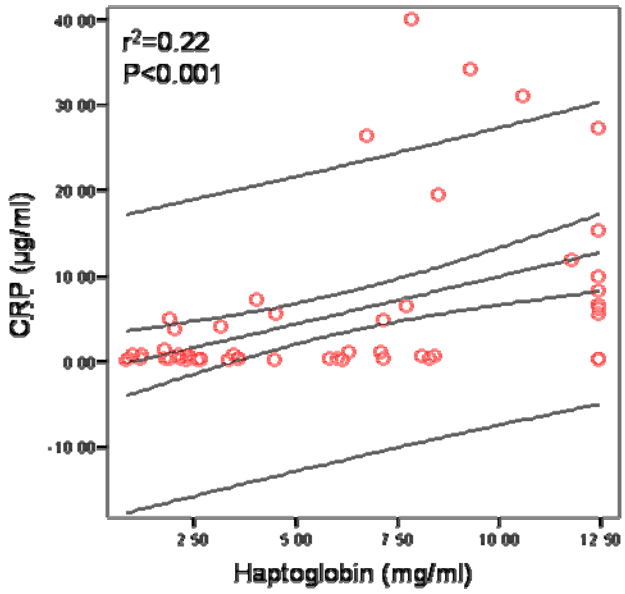


Figure 5.5 Single regression analysis between haptoglobin and CRP

Haptoglobin was significantly correlated to C-reactive protein (P <0.001). The central straight line corresponds to the best-fit linear regression line. The two curved lines surrounding the best-fit line define the 95% confidence interval of the regression line. The graphs also show the prediction interval (the curves defining the prediction interval are further from the regression line than the confidence lines).

5.3 Development of a novel intra-operative diagnostic kit for detection of ovarian malignancy using cyst fluid

Aim. Regular pelvic examinations and CA-125 biomarker measurements followed by radiological diagnosis on an individualised basis have been the current practice for detection of ovarian cancer. However, the impact of screening on mortality from ovarian cancer remains to be a daunting challenge. In contrast, the impact of accurate staging and appropriate surgical intervention for all stages of EOC on patient survival is clear since the mortality risk is twice for patients who do not undergo surgery (Engelen et al., 2006).

The study by Engelen et al (2006) showed that inappropriate surgical management of the patient adversely affects the patient's chances of survival. Appropriate surgical management is largely dependent on the intra-operative diagnosis of benignity or malignancy of the ovarian cyst, making correct intra-operative diagnosis critical to proper patient management. Moreover, an ovarian malignancy is unexpectedly encountered in up to one percent of cases undergoing laparoscopy (Wenzl et al., 1996; Muzii et al., 2005). Neither an elevated serum CA-125 level, nor the presence of an ovarian cyst identified by clinical examination and ultrasonography, accurately predicts the occurrence of an ovarian malignancy (van Nagell et al., 2000). Frozen section of ovarian cyst tissue is the currently used method for intra-operative diagnosis, but it is expensive and resource intensive and requires the services of a trained pathologist or gynaecologic oncologist. Frozen section diagnosis of ovarian tumours has also been reported to be most problematic in terms of its accuracy (Boriboonhirunsarn et al., 2004). In addition, such services are not available in many developing countries. A better alternative to this method would clearly be

very useful, and this was the rationale for developing this intra-operative diagnostic kit.

We envisaged the possibility of using biological materials obtained at surgery to identify specific protein biomarkers which could be utilised in the diagnosis of EOC for subsequent triage of frozen section. These may include tissue, ascitic and ovarian cyst fluids. It is noteworthy that the cyst fluid represents a suitable, evenly diluted and consistent source of intra-operative biological material which could be readily examined in the operating theatre for the presence of biomarkers of interest. Using ovarian cyst fluid it has been observed that differences in hormone levels, such as LH and FSH (Chudecka-Glaz et al., 2004; Rzepka-Gorska et al., 2004), potential tumour markers including tissue polypeptide specific antigen and soluble interleukin-2 receptor-alpha (Sedlaczek et al., 2002) and differences in proteomic profiles occurred between benign and malignant ovarian cysts (Ott et al., 2003). However, none of the above mentioned markers could be utilised clinically to discriminate between benign and malignant ovarian cysts.

Data from this study indicated that haptoglobin levels were significantly lower in benign when compared with borderline, early and late stage EOCs ($P<0.01$). We hypothesised that a rapid intra-operative detection assay could be developed using this protein as an adjunct to the frozen section technique for identification of malignancy.

Investigation. A systematic strategy to identify unique biomarkers within the cyst fluid was employed resulting in the identification and characterisation of haptoglobin in cyst fluid from EOC as described earlier (chapters 3 and 4).

Workflow of preliminary studies

Step 1	SELDI-TOF protein profiling of cyst fluid samples from benign and malignant ovarian cysts
Step 2	Gel electrophoresis to identify differentially expressed proteins
Step 3	Mass spectrometry identification of differentially expressed protein
Step 4	Western blotting and SELDI-immunocapture to confirm identity of protein
Step 5	Development of sandwich ELISA method for haptoglobin assay
Step 6	Immunohistochemistry to confirm tissue specific expression of haptoglobin in malignant samples
Step 7	Testing commercial dye assay method for haptoglobin
Step 8	Testing efficacy of the assay in stored cyst fluid samples
Step 9	Combining clinicopathologic variables
Step 10	Development of a diagnostic kit for rapid intra-operative haptoglobin detection

To determine the effectiveness of an accurate and rapid method for detection of malignancy in ovarian tumours, the cyst fluid haptoglobin binding assay was conducted in a low pH environment using a commercial PHASE RANGE haptoglobin assay kit (Tridelta Development Ltd; section 2.2.13). This semi-quantitative method permits the determination of this acute phase reactant readily in the operating theatre. Pearson's correlation analysis was also performed to evaluate the correlation between the levels of haptoglobin between the PHASE RANGE assay and the ELISA technique. The diagnostic accuracy of this biomolecule together with other clinicopathologic variables such as ultrasound and CA-125 was evaluated using the ROC curve analysis to differentiate malignant from benign ovarian tumours.

Using this strategy, a diagnostic device was developed for the rapid intra-operative haptoglobin detection. In the first phase of this study we designed a point-of-care device which could contain the dye binding assay reagents within it. This device must fulfil the following criteria: it must be easy to perform by the

nurse in the operating theatre; it must contain the appropriate amount of the reagents used in the dye binding assay and finally it must be small, inexpensive and readily portable unit which can be stored conveniently in the refrigerator.

Results and interpretation. The colour reactions obtained at 5-minute time point for the PHASE RANGE assays using cyst fluid from benign and malignant ovarian tumours are illustrated in Figure 5.6. As indicated in Figure 5.7, a good correlation was observed between the PHASE RANGE haptoglobin assay colour intensity and levels of haptoglogin measured by the ELISA technique, with a Pearson's correlation coefficient r square value of 0.79. Measurements of cyst fluid haptoglobin by the 5-minute colour reaction using the PHASE RANGE assay would be potentially useful in the differentiation of benign from EOCs.

Comparison of frozen section with final pathological diagnosis was made and the misdiagnosed cases were highlighted in Table 5.3. It was envisaged that this rapid assay could accurately detect the two misdiagnosed borderline tumours as indicated in Table 5.3. ROC curves for haptolgobin, ultrasound and CA-125 were then conducted to examine the ability of these parameters to differentiate malignant from benign ovarian tumours. It showed that haptoglobin had an enhanced predictive performance when combined with CA-125 and ultrasound parameters giving an AUC of 0.946 with a 95% confidence interval of 0.900-0.992 (Figure 5.8). Moreover, a combination of the three parameters in our patients was observed to have a sensitivity of 88.4%, a specificity of 91.5%, a PPV of 90.5%, and a NPV of 89.6% in detection of ovarian cancers.

This rapid assay method could therefore be used in established oncology centres to triage for frozen section or trigger appropriate intraoperative subspecialist consult and ensure best practice care.

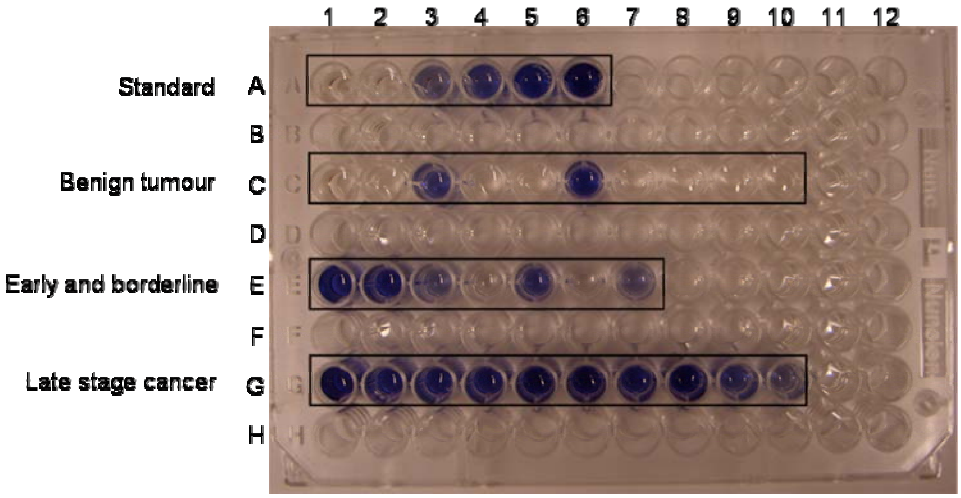
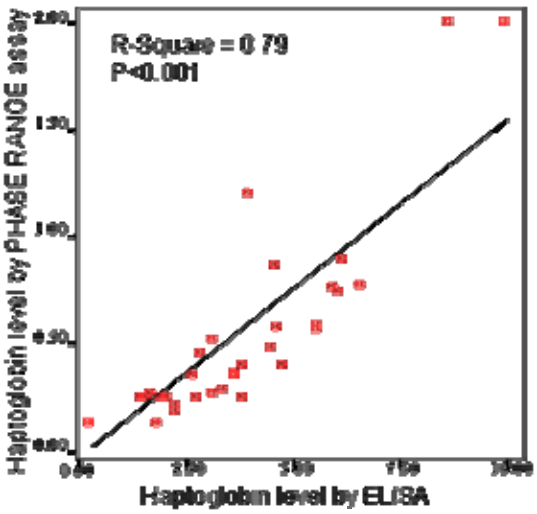


Figure 5.6 Representative picture of the haptoglobin dye binding assay showing standards, benign ovarian tumours, early and late stage ovarian cancer samples

A1: blank; A2-A6: Standards (0, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml); C1-C10: benign ovarian tumours; E1-E7: early and borderline ovarian tumour; G1-G10: late stage ovarian cancer.

Table 5.3 Cases with disagreement between frozen section and final paraffin diagnosis

ID	Pathological diagnosis	Frozen section	Colour reaction
59	Mucinous borderline tumour	Mucinous cystadenoma	**Positive**
128	Mucinous adenocarcinoma	Mucinous borderline	Negative
133	Serous adenocarcinoma	Borderline	**Positive**
161	Serous adenocarcinoma	Serous borderline	Negative
166	Serous adenocarcinoma	Serous borderline	Negative
182	Mucinous borderline	Mucinous cystic tumour	**Positive**



Haptoglobin level = -0.02 + 0.16 * Haptoglobin level (ELISA)

Figure 5.7 Single regression analysis between haptoglobin levels measured by ELISA and the PHASE RANGE assays
Haptoglobin levels from ELISA was significantly correlated with that of the PHASE RANGE assays (r^2=0.79, P<0.001)

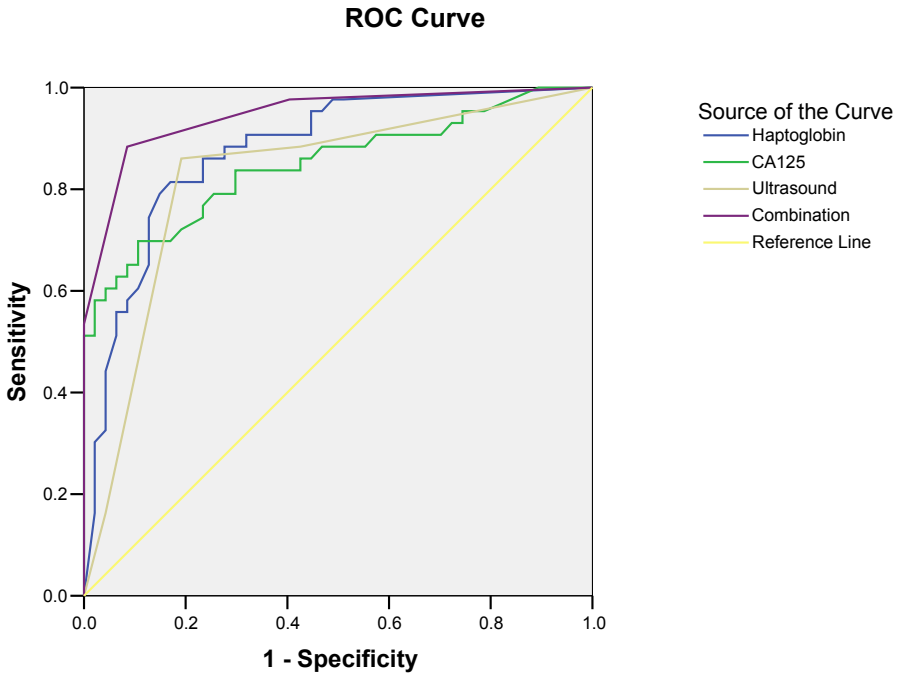


Figure 5.8 ROC curve of ultrasound, CA-125 and haptoglobin assay
Haptoglobin enhanced the diagnostic value of ultrasound and CA-125 (AUC 0.946, 95% CI 0.900-0.992). Combination of all three parameters gave a sensitivity of 88.4%, a specificity of 91.5%, a PPV of 90.5%, and a NPV of 89.6% for ovarian malignancy.

Development of diagnostic kit: (*Confidential*)

A prototype of the diagnostic kit which applies the principle of Phase Range Haptoglobin assay into real time testing of intra-operative levels of haptoglobin in cyst fluid has been developed. As indicated in Figure 5.9, the device comprises an assembly of two parts: the top portion having four extruding, reagent-containing plastic bubbles and the bottom part having reagent-passage channels engraved on a plastic base. The technical details of this diagnostic kit are as follows:

a) 100 µl of diluted haemoglobin solution, 50 µl of substrate and 90 µl of chromogen from a commercial PHASE RANGE Haptoglobin assay kit (Tridelta Development Ltd) will be injected into bubbles B2, B3 and B4 from the injection holes on the base, sealed properly and stored at 4 °C.

b) In the operating theatre, the nurse will press bubble B2 to allow diluted haemoglobin solution to flow into the empty bubble B1, apply 7.5 µl of cyst fluid from patient into bubble B1 through the application hole, press bubble B3 to release substrate into chromogen in bubble B4, and finally press bubble B4 to allow substrate-chromogen mixture flowing into bubble B1. This will be followed by incubation for 5 min at room temperature.

c) Haptoglobin present in the cyst fluid will bind with haemoglobin and at low pH the peroxidase activity of the bound haemoglobin will be preserved. Addition substrate and chromogen will result in a colorimetric reaction which can be determined either visually or by the ELISA reader at 630 nm. Preservation of the peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin present in the cyst fluid.

d) The blue coloration which develops after 5 min will be recorded and compared with a standard cut-off colour scheme to differentiate malignant from benign ovarian tumour.

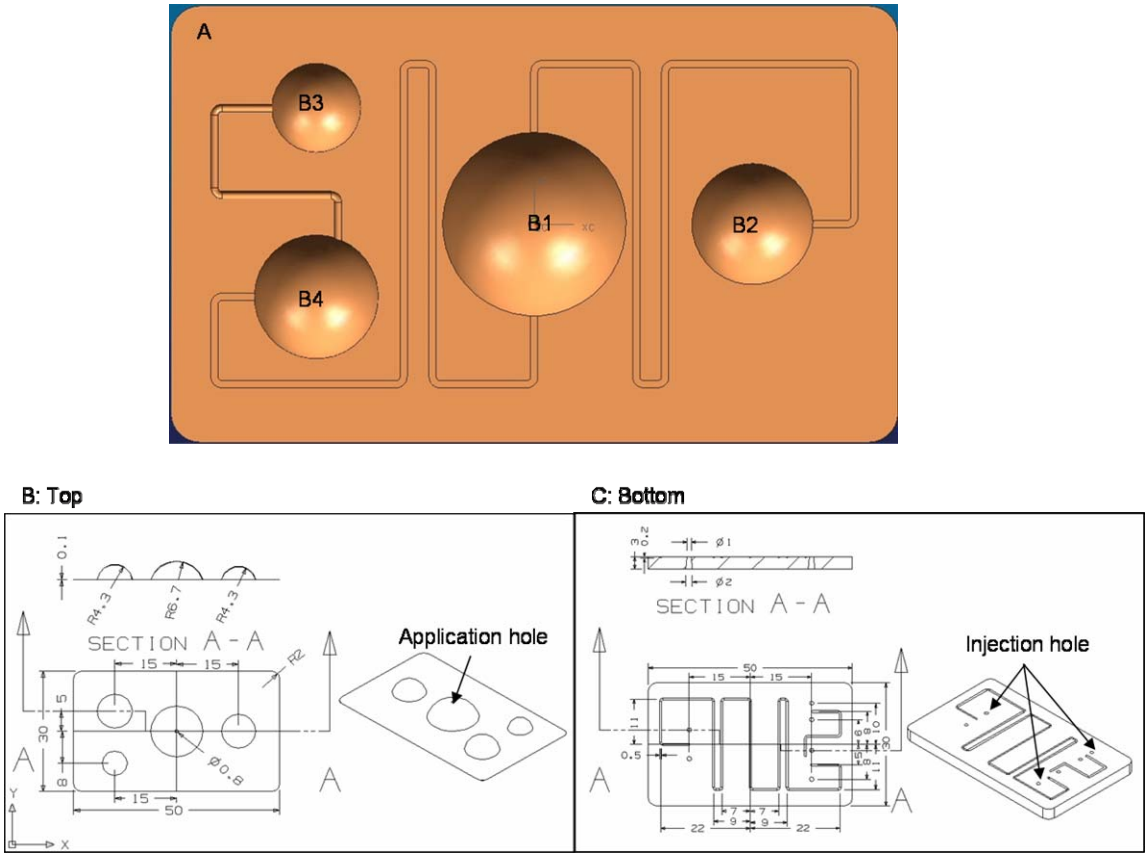


Figure 5.9 Demonstration of designation of the portable device

5.4 Conclusion

In this chapter, clinical application of the identified protein biomarker haptoglobin from SELDI-TOF and 2-DE based methods was validated in sera and cyst fluid from EOCs. It was observed that levels of haptoglobin were significantly higher in malignant when compared with benign ovarian tumours. Prognostic analysis using uni- and multivariate analyses indicated that circulating haptoglobin levels could be a useful independent prognostic indicator in patients presenting with EOCs. Moreover, a haptoglobin assay was also evaluated for its usefulness to triage malignant from benign ovarian cysts. It is envisaged that this newly developed PHASE RANGE assay device could complement the frozen section technique and could therefore be used in established oncology centres to triage for frozen section or trigger appropriate intraoperative subspecialist consult and ensure best practice care. More studies are in progress to conduct multicentre trials in South East Asia with a view to establish a potential role for haptoglobin in detecting EOCs.

Chapter 6: General Discussion

Investigations on proteomics-based identification of biomarkers were conducted in ovarian cyst fluid. This fluid represents an ideal source for biomarkers in that it is constantly in contact with the epithelial cells forming the cyst wall, so the protein composition changes happening in ovarian epithelial cells as they become malignant will be reflected in the cyst fluid in the undiluted form. Therefore, cyst fluid was regarded as a suitable and consistent source of biomarker identification as well as intra-operative biological material that could be readily examined in the operating theatre.

We hypothesised that a comprehensive analysis of cyst fluid proteins using a combination of high throughput technologies including SELDI-TOF and 2-DE might provide a sequence-based network for the mining of potential polypeptides which could serve as diagnostic and prognostic targets.

Based on this premise, the main objective of this work is to identify differentially expressed protein markers in the cyst fluid from patients presenting with benign and malignant ovarian tumours. The diagnostic and/or prognostic potential of these biomarkers was further evaluated in the clinical setting. As detailed in chapters 3 and 4, the possibility of identification of differentially expressed proteins in cyst fluid using SELDI-TOF and 2-DE based methods was examined. The diagnostic and prognostic implications of identified biomarkers within the field of epithelial ovarian tumours were then described in chapter 5.

6.1 Hypotheses

The aim of this thesis was to identify potential diagnostic and/or prognostic protein markers using the cyst fluid as a model. A combination of two proteomics-based methods SELDI-TOF and 2-DE were utilised in this study in searching complimentary biomarkers. I hypothesised that 1) differential disease related proteins may be present in cyst fluid from benign and malignant ovarian tumours and could be identified using a combination of SELDI-TOF, 2-DE and conventional immunological techniques; 2) Similar protein patterns may also be observed in sera from benign and malignant ovarian tumours and they may have potential roles as diagnostic and/or prognostic markers; 3) An intra-operative diagnostic assay may be developed using cyst fluid to enable the differentiation between malignant and benign ovarian tumours at the time of surgery.

6.2 Research findings

The expression profile of proteins in cyst fluid from patients presenting with benign and malignant epithelial ovarian tumours was examined using different proteomics-based technologies including SELDI-TOF and 2-DE. As indicated in chapter 3, a protein peak with molecular weight around 17.5 kDa was present with higher intensities in EOCs when compared with controls using SELDI-TOF (section 3.2). This protein was purified using SDS-PAGE and identified as haptoglobin-α_2 subunit by MALDI TOF-TOF/MS (section 3.3). Using 2-DE as a complementary method, the differentially expressed protein spots were highlighted and identified as two acute phase proteins namely, haptoglobin and ceruloplasmin (section 4.3). Further validation of these putative biomarkers using variable immunologic techniques including Western blotting,

immunohistochemistry and ELISA confirmed our findings that differential expression of haptoglobin occurred in ovarian cancer (section 4.4).

Our data therefore consistently demonstrated that haptoglobin was overexpressed in malignant ovarian tumour samples including cyst fluid, tissue and serum samples. Serum haptoglobin levels were significantly elevated in ovarian cancer patients with advanced-stage disease or poor performance status (section 5.2). Moreover, a simple and rapid intra-operative diagnostic kit using cyst fluid was developed for detection of patients presenting with malignant ovarian tumours (section 5.3). A preliminary study using this PHASE RANGE assay on 90 ovarian tumour samples indicated an effective diagnostic power capable of differentiating malignant from benign ovarian tumours. It showed that haptoglobin had an enhanced predictive performance when combined with CA-125 and ultrasound parameters giving 88.4% sensitivity and 91.5% specificity with a PPV of 90.5% and NPV of 89.6% for EOCs.

The molecular mechanisms underlying carcinogenesis and metastasis of EOCs remain obscure at present. It is noteworthy that the two molecules identified in this study could provide us knowledge to better understand the pathological processes involved in the progression of EOCs. Data from this study have demonstrated for the first time the presence of haptoglobin which has the potential to serve as a marker in cyst fluid from patients presenting with EOC. The basic haptoglobin molecule is a tetrameric protein with two α/β dimers. The β chains are identical in all haptoglobin types and the polymorphisms of haptoglobin arise from differences in the α chains (Gerner-Smidt et al., 1978). Although the biological functions of haptoglobin are not well understood, recent data suggest that this glycoprotein possesses specific physiological activity distinct from its role as a haemoglobin scavenger following haemolysis, thereby

preventing iron loss, renal damage and tissue destruction (Wassell, 2000). Haptoglobin is also a potent antioxidant which protects low density lipoprotein from copper induced oxidation thereby having a role in cellular resistance to oxidative stress (Tseng et al., 2004b). In addition, this acute phase protein has been shown to be involved in the regulation of epidermal cell transformation (Xie et al., 2000), immune suppression in cancer(Oh et al., 1987; Oh et al., 1990) and as an angiogenic factor in systemic vasculitis (Cid *et al* 1993). Moreover, haptoglobin was reported to be a natural inhibitor of collagen degradation and locally expressed by fibroblasts in the arterial wall (Smeets et al., 2002). This glycoprotein could therefore serve an important role in cell migration and arterial restructuring. This collagen turnover is an important feature in many physiological processes like growth and wound healing and enhanced collagen degradation is causally related to severe tissue destruction and malfunction as evidenced by known pathophysiological processes such as arthritis and cancer (Westermarck et al., 1999). The importance of haptoglobin in extracellular matrix degradation and cell migration suggests a role for this polypeptide in the aetiology of cancer.

Elevation of haptoglobin in sera and ascites of ovarian cancer have also been reported in previous studies using the ELISA method (Dobryszycka et al., 1991; Elg et al., 1993). Recently, proteomics-based approaches have been utilised to discover and identify novel proteins as potential diagnostic biomarkers. Ye and coworkers have shown that haptoglobin α-subunit as potential serum biomarker in ovarian cancer with a sensitivity of 95% and a specificity of 91% combined with CA-125 (Ye et al., 2003). Another recent study found that haptoglobin-1 precursor as a novel circulating biomarker of ovarian cancer (Ahmed et al., 2004). Nevertheless, in this study, we provide the evidence that pre-operative serum levels of haptoglobin could serve as an independent prognostic factor in patients

presenting with EOC (Zhao et al., 2007). We also developed a rapid intra-operative diagnostic kit, a device that could complement the frozen section technique and differentiate malignant from benign ovarian tumours in operating theatre (section 5.3).

In addition to haptoglobin, ceruloplamsin was also noted to be differentially expressed in ovarian cyst fluid and tissues from EOCs using semi-quantitative analysis. However, in this study, no significant difference of cyst fluid ceruloplasmin concentrations was discernible between early, late stage ovarian cancers and in benign ovarian tumours using ELISA method (section 4.4.5), which limited its usage as a useful biomarker in clinical settings.

6.3 Significance of this study

Prognostic significance of circulating haptoglobin levels

In this study we demonstrated for the first time the differential expression and secretion of haptoglobin and ceruloplasmin in cyst fluid from EOCs using complementary proteomics-based methods. Our retrospective analysis demonstrated that circulating haptoglobin levels therefore could be a useful independent prognostic indicator in patients with EOC. It is noteworthy that significantly elevated haptoglobin concentrations were associated with poor outcome for survival in our patients. Early recognition of a profile with high haptoglobin levels could therefore identify patients with an increased risk of poor prognosis for a better treatment adaptations based on this knowledge.

Intra-operative diagnosis of EOC with a diagnostic kit using cyst fluid

The initial evaluation using a simple and rapid intra-operative diagnostic kit on 90 ovarian tumour samples indicated an effective diagnostic power capable of differentiating malignant from benign ovarian tumours based on differential cyst fluid haptoglobin levels. This kit is both easy for nurses in the operating theatre to use, and also give the colorimetric result that can be easily interpreted. We have also designed a portable device (Figure 5.9) which could be safely stored and easily transported to other countries so that this kit could be applied in other hospitals upon satisfactory results from further validation studies. This finding has potential clinical significance in that it could be performed as an adjunct to frozen section and be utilised to triage women requiring frozen section or sub-specialist consult, so that these services are more cost-efficient.

6.4 Limitations and future directions

Identification of low abundant proteins using more sensitive proteomics based methods

Cellular proteins that are overexpressed by the malignant cells and released into the bloodstream during cancer development are ideal biomarkers and they can be used alone or in combination with other established tumour markers for diagnosis and prognosis of cancers. These markers are usually present in serum at relatively low concentrations of 1–10 ng/ml in the normal state (Erika, 2004). In this study, however, we are not able to identify such low abundant biomarkers in serum other than haptoglobin. It is possible to speculate that low abundance proteins may be diluted or degraded after being released into the circulation. In this regard, growth factors and cytokines are a group of molecules and have been shown to play important roles in the development and growth of various

kinds of cancers (Aaronson, 1991; Cross et al., 1991) and it has been proposed that EOC may be a cytokine propelled disease (Malik et al., 1991). Our preliminary results have revealed that a novel proinflammatory cytokine—Interleukin 22 (IL-22) was differentially expressed in benign and malignant ovarian tumours (Figure 6.1). A more detailed study of the various cytokines and growth factors could therefore provide a panel of sensitive and specific candidate protein markers for EOCs. More sensitive technologies such as 2D-DIGE, ICAT, iTRAQ and antibody-based protein arrays should therefore be employed to detect minute quantities of such proteins from the patient proteome. Moreover, pooled-sample testing has proved to be an effective strategy to increase the power and reduce the cost of monitoring populations for low-prevalence diseases (Ogata et al., 2007). This strategy may provide stability in protein detection and also reduce inter-sample variations.

Validation of clinical significance of haptoglobin in a multi-centre study

It is envisaged that several criteria have to be met before biomarkers can be utilised for clinical practice, such as preclinical evaluation, assay development and clinical validation, retrospective analysis and prospective screening (Pepe et al., 2001; Barker, 2003). Based on these preliminary findings, validation of the results are therefore necessary for conducting prospective, well-controlled clinical trials with a larger sample size and stringent standards for all procedures. Considering the varied protocols employed by the different research centres, more comprehensive standardisation procedures are required in order to achieve reliable and reproducible results. This problem could be circumvented by multi-institutional teamwork, through participation in large collaborative oncology groups, which may advance both analytical and clinical validation by creating standards and experimental designs in a bid to use the limited patient samples and resources most effectively (Ludwig et al., 2005). The gynaecological

oncology centres in the Asian countries have indicated their interest to participate in this multi-centre collaborative effort. The initiation of the Ovarian Cancer Research Group or Consortium in our centre will further enhance our efforts to contribute towards biomarker discovery in ovarian cancer research. This collaborative effort is the first of its kind in Asia and efforts are being made initially to include centres from Indonesia and Malaysia where there is an increasing incidence of this cancer in women.

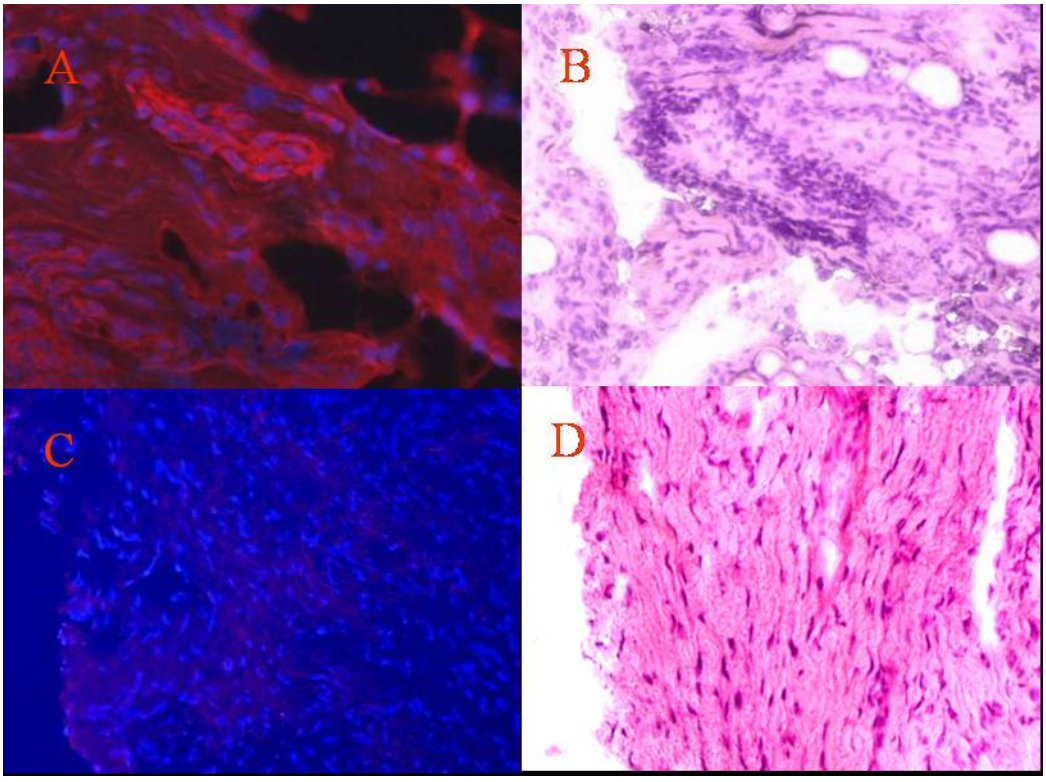


Figure 6.1 Differential expression of IL-22 in ovarian tumour tissue
IL-22 is highly expressed in invasive ovarian cancer (A) compared with benign ovarian tumour (C) using immunofluorescent method, corresponding H & E staining was also showed (B, D) original magnification 200×

6.5 Conclusion

The main aim of this study is to identify differentially expressed biomarkers in cyst fluids from benign and malignant ovarian tumours. In order to facilitate the biomarker identification, two complementary proteomics based methods including SELDI-TOF and 2-DE were utilised. Data from this study provides strong evidence that the differential disease related protein haptoglobin was present in cyst fluid and serum from ovarian tumour patients with diagnostic and prognostic potentials, which could be explored using combination of proteomics-based methods. Circulating haptoglobin identified could have potential significance as a prognostic marker for EOCs. Moreover, the diagnostic kit developed in this study based on differential cyst fluid haptoglobin levels might be used intra-operatively to differentiate malignant from benign ovarian tumours so as to triage for frozen section or trigger appropriate intraoperative subspecialist consult and ensure best practice care. Thus, this thesis suggests that proteomics-based identified haptoglobin is a useful biomarker for EOC.

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Appendix: Publications

Changqing Zhao, Loganath Annalamai, Narasimhan Kothandaraman, Stephen Chee Liang Koh, Huoming Zhang, Arijit Biswas and Mahesh Choolani. Circulating haptoglobin is an independent prognostic factor in sera of patients with epithelial ovarian cancer. Neoplasia 2007, 9(1), 1-7.

Huoming Zhang, Qingsong Lin, Sukumar Ponnusamy, Narasimhan Kothandaraman, Teck Kwang Lim, **Changqing Zhao**, Mahesh Choolani et al. Proteomic analysis of human erythrocyte membrane proteins extracted using methanol and trifluoroethanol. Proteomics 2007, 7(10): 1654-1663.

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Changqing Zhao, Loganath Annalamai, Narasimhan Kothandaraman, Stephen Chee Liang Koh, Huoming Zhang, Singini Biswas, Ilancheran A., Khalil Razvi, Arijit Biswas and Mahesh Choolani. Proteomics-based Identification of Haptoglobin in Cyst Fluid: A Potential Diagnostic Marker for Epithelial Ovarian Cancer. Manuscript in preparation.

Changqing Zhao, Loganath Annalamai, Narasimhan Kothandaraman, Stephen Chee Liang Koh, Huoming Zhang, Khalil Razvi, Arijit Biswas and Mahesh Choolani. The differential expression of proinflammatory cytokine IL-22 in cyst fluid from benign and malignant ovarian tumours. Manuscript in preparation.

Huoming Zhang, Sukumar Ponnusamy, **Changqing Zhao**, Biswas Arijit., Mahesh Choolani. CD147, a potential marker for the enrichment of first trimester erythroblasts from maternal blood. Manuscript in preparation.

Huoming Zhang, Qingsong Lin, Sukumar Ponnusamy, Narasimhan Kothandaraman, **Channgqing Zhao**, Mahesh Choolani. Proteomic analysis of human primitive erythroblast membrane proteins. Manuscript in preparation.

Mahesh Choolani, Khalil Razvi, Arijit Biswas, Loganath Annalamai, Narasimhan Kothandaraman and **Changqing Zhao**. Diagnostic molecules, patent reference No. SP7665 (pending).

Changqing Zhao, Singini Biswas, Lin Liu, Narasimhan Kothandaraman, Stephen Chee Liang Koh, Arijit Biswas, Mahesh Choolani. Proteomics-based identification of haptoglobin in cyst fluid: a potential diagnostic biomarker for epithelial ovarian cancer. Oral presentation at the Sixth Obstetrics and Gynaecology Society of Singapore Congress, Singapore, March 21-25, 2007. Abstract book page 155.

Changqing Zhao, Loganath Annalamai, Narasimhan Kothandaraman,Huoming Zhang, Mahesh Choolani. Identification of potential protein markers for epithelial ovarian cancer using proteomics-based methods. First Obstetrics and Gynaecology Research Fair, Singapore, 3 -4 March, 2007. (Prize for **Best Oral Presentation**, Clinical Research Category).

Changqing Zhao, Loganath Annalamai, Mahesh Choolani et al. Proteomics-based identification of differentially expressed proteins in cyst fluid from benign and malignant epithelial ovarian tumours. Joint Third AOHUPO and Fourth Structural Biology and Functional Genomics Conference, NUS, Singapore, 4-7 December 2006.

Changqing Zhao, Loganath Annalamai, Mahesh Choolani et al. Effect of serum haptoglobin level on survival in patients with epithelial ovarian cancer. National Healthcare Group Annual Scientific Congress, 30 Sep-1 Oct 2006. Ann Acad Med Singapore 2006; 35 (suppl) 10: S5. (Prize for **Best Poster Presentation** in the Clinical Research Session).

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