LC-MS/MS proteomics analysis of biomarkers in Singapore breast cancer patients on doxorubicin treatment and MCF7 breast cancer derived cells

Bahareh Haji Rasouliha

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**A LC-MS/MS PROTEOMICS ANALYSIS OF
BIOMARKERS IN SINGAPORE BREAST CANCER
PATIENTS ON DOXORUBICIN TREATMENT AND MCF7
BREAST CANCER DERIVED CELLS**

BAHAREH HAJIRASOULIHA

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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Abstract

Breast cancer is known as the most common cancer in women population, especially in developed countries. Improved prognosis and effective treatment are needed to reduce the mortality of the disease. Doxorubicin, a topoisomerase II-targeting chemotherapeutic drug has been widely used to treat breast cancer. The rapidly evolved proteomics techniques, especially LC-MS/MS analysis, has been used in protein profiling in body fluids as well as cells, which provide us with a deep comprehension in drugs mechanism and patients response to the treatment. This new method can be extended in biomarker identification to improve diagnosis and monitoring of treatment.

Effective treatment of breast cancer by Doxorubicin has been complicated by the single nucleotide polymorphism (SNP 309) in MDM 2 gene. The polymorphism is caused by substitution of Guanine over Thymine in its promoter. SNP 309 can be homozygous (GG) or heterozygous (TG) with different frequency in the general population. This polymorphism promotes tumor susceptibility and resistance to topoisomerase II-targeting agents.

We aim to identify plasma proteins which correlate with a particular SNP309 genotype by using LC-MS/MS proteomics approach. The main objective of this research is to find the effective expressed biomarkers which should facilitate the monitoring of Doxorubicin treatment. Two different targets were selected in this study: 1) thirty breast cancer patients' on Doxorubicin treatment; 2) breast cancer-derived MCF7 cell line incubated with Doxorubicin. The plasma samples from the patients selected in this study were separated in three groups based on MDM2 single nucleotide polymorphism.

The identified proteins from plasma samples with heterozygous (TG) and homozygous (GG) mutant genotypes were categorized in two groups: up-regulated proteins and down-regulated proteins were identified with significant ($p < 0.05$) changes from the view of statistic. In the GG genotype group of samples, three proteins including α -1-antitrypsin, ceruloplasmin and Serotransferrin were up-regulated while hemoglobin subunit β and α 2 macroglobulin were down regulated. In TG genotype group of samples, Serotransferrin, Apolipoprotein A-1 and Clusterin were down-regulated whereas ceruloplasmin were up regulated.

The protein profiling MCF7 cells incubated with Doxorubicin as compared with untreated MCF7 cells indicated that 10 cellular proteins were down-regulated and 13 others up-regulated. These included HSP 90, glyceraldehyde-3-phosphate dehydrogenase, peroxiredoxin-1, and Calreticulin precursor. It is to be hoped that proteins identified in our study can be candidate biomarkers in monitoring Doxorubicin therapy in patients.

1. Introduction

Breast cancer is considered as the first ranked death cause in women. Regarding high rate of death in this kind of cancer, reliable and fast diagnosis methods and treatment monitoring approaches are required as well as effective and useful therapy.

In this project, we performed LC-MS/MS proteomics analysis to identify potential biomarkers in breast cancer patients' plasma on Doxorubicin treatment, as well as MCF7 cell line incubated with Doxorubicin, a topoisomerase II-targeting chemotherapeutic drug. Our plasma samples are classified in three groups based on MDM2 single nucleotide polymorphism. We propose to study the correlation of MDM2 mutation and response of breast cancer patients to Doxorubicin by using LC-MS/MS proteomics approach. The main objective of this research is to find effective expressed biomarkers which should facilitate the monitoring of Doxorubicin treatment.

1.1 Breast Cancer

Based on ACS (American Cancer Society) agreement, cancer is referred to as a disease characterized by the uncontrolled growth and dividing of the mutated or abnormal cells. During damage normal cells accelerate their cell division and reproduction until the injury is recovered. When the cancer cells become invasive they devastate the part of the body they originated and then extend to the other parts which means metastasis. In contrast, benign tumors remain confined and even though the cells may grow and divide fast [1].

Breast cancer is a genetic disorder results in a series of genetic changes in normal mammary epithelial cells to transform them into cancerous type. Two major genes, oncogenes and tumor suppressor genes (TSG) play important role in cancer cell formation [2]. The abnormal activity of oncogenes which, may emanate from chromosomal translocations, point mutations or gene amplification leads to cancer formation. From the other hand, the inhibition of tumor suppressor genes that naturally prevent the cancer is caused by chromosomal deletions or point mutations. In recent years, epigenetic modification and methylation of gene promoter is considered as another genetic cancer causes. Both TSG and oncogenes are interwoven together and mainly affect on some fundamental processes in the cell including differentiation, cell growth and cell death. However, 5-10% of breast cancers are originally hereditary and raised from special mutations in some TSGs such as P53, BRCA1, and BRCA2 [2]. This disease appears in various forms including malignant and swift progression with lethal futurity while other types might be benign and non-invasive without progression. However, the early detection by sensitive and accurate screening methods is needed to allow early intervention and thus decrease the mortality.

Since 1989, better basic understanding of breast cancer has been documented. Together with newly discovered screening methods and anticancer drugs, the rate of mortality has remarkably reduced. However, this disease is still the most common cancer in women and the second death cause of cancer in the world [3]. Based on a statistics analysis in 2002, worldwide breast cancer patients are at 1.5 million [4], while in Europe and North America the rate incidence was estimated to be 36100 and 230000, respectively [5]. In

addition, Weir et al. showed that the rate of breast cancer death is high in Blacks and Whites comparing with other races such as Hispanic, American Indian/Alaska Native and Asian/ Pacific Islanders [6]. The rate of mortality and incidence has shown in Figure 1.

Figure 1. The Rate of Breast Cancer Incidence and Mortality [6]

The most and the least breast cancer incidence has been illustrated in Whites and Asian/Pacific Islanders, respectively. The highest rate of death has been seen in Blacks and Whites. Age-Standardized Rate is per 100,000

The geographical distribution and differential incidence rate of breast cancer are summarized in Figure 2.

Figure 2. Breast Cancer Incidence in the World ^[7]

Highest incidence rate is seen (shown by red) in northern America, Western Europe, Australia and New Zealand. While, the lowest incidence rate (dark green) is related to some part of Africa, Middle East, and Far East including China.

Effective treatment is a key factor in patients' survival. Most of recent therapeutic agents are not as effective as they are supposed to be. Moreover, patients' reaction to the treatment can be different based on other factors including genetics, immune system and diet habits. Monitoring the efficiency of chemotherapy is a critical step in controlling the disease progression. Early diagnosis, monitoring and finding the best treatment would lead to reduction of serious side effects of chemotherapeutic agents and the mortality of this disease.

Among putative risk factors contributing to breast cancer development, 5-10% of cases are associated with inheritance. However, hormones and reproducibility factors including the age of puberty, marriage, first birth, parity and menopause are also important. These items determine exposure of lifetime to estrogen [8]. The other effective factors in breast cancer incidence are environmental factors such as ionizing radiations or polyaromatic hydrocarbons and high fat diet.

1.2 Proteomics

Proteomics is referred to the global analysis of expression of proteins. This method includes protein detection and investigating the roles of these proteins in physiological and pathological functions of any organism [9, 10]. Cells are made of proteins with specific function that expressed in different conditions [11]. A comparative analysis of the protein profile can help us identify proteins involved in special disease development or indicative of treatment efficiency. In this method proteins are fragmented into smaller peptides. By using mass spectrometry the weight of each fragment is measured that leads

to protein identification [12]. This approach is useful to analyze proteins from different samples including tissues, cells, plasma and serum that are expressed in various environmental and physiological circumstances [13]. There are different proteomics tools combining of mass spectrometry (MS) to detect proteins with high accuracy, sensitivity and specificity and various systems such as liquid chromatography, two dimensional electrophoresis, microarray and strong cationic exchange that are applied for biomarker discovery purposes. Each of which has pros and cons that will discussed in next parts of this project [14]. In Figure 3, three different “Omics” family including genomics, transcriptomics and proteomics are shown [9].

Figure 3. A Simple Comparison of Genomics, Transcriptomics and Proteomics

1.2.1 LC-MS/MS

LC-MS/MS is a proteomics method combined of a soft ware, liquid chromatography and mass spectrometry that is an approach to detect peptides as biomarkers. In mass spectrometry part, proteins are broken down into smaller fragments like peptides or amino acids according their molecular weight/charge (m/z) after digesting by trypsin. Three major factors [13, 15] including m/z , intensity and time are considered in this system [16]. Whereby a fragment with a m/z ratio will be identified with a special intensity at a particular time [16].

1.3 Biomarker

Biomarkers are protein-based components including, enzymes, immunoglobulins and any indicated protein as a marker that could be applied to identify and monitoring different stages of a disease as well as treatment process [17]. Moreover, biomarkers should have enough sensitivity and specificity to avoid of some false positive or negative results. They are considered as indicators that show normal or pathogenic conditions and even pharmacologic reaction of body during the treatment. Identification of these components may result in early detection, monitoring the disease and analysis the response to therapy [18].

In biomarker discovery field, two key words called up and down regulation are seen frequently. A down regulation is defined as when the expression of a gene is decreased, it results in lower level of specific protein which is encoded by this gene. On the other hand,

an up regulation defined as highly generating of protein following over expression of corresponding gene. Moreover, the criteria respect to up and down regulation for protein expression is discussed in page 63 and 75 of result and discussion part.

So far, a few biomarkers such as CA15.3, CA 125 and CEA have been discovered for breast cancer detection in clinical experiments [19, 20] but they suffer from loss of predictive and valuable affects. It is therefore important to identify new biomarkers for early diagnosis as well as treatment monitoring [21].

1.4 MDM2

Murine Double Minute2 is a gene that releases a phosphoprotein in the nucleus with the same name. This gene is known as a negative regulator for a principle tumor suppressor gene, P53, by deactivating of P53-dependent transcription [22]. P53 and MDM2 have an autoregulatory feedback so that MDM2 affects negatively on P53 activity whereas P53 increases the level of MDM2 [23]. Following DNA damage, both MDM2 protein and mRNA levels are dropped. Subsequently, transcriptional activity of P53 will be raised and leads to an accretion in P53 levels. High activity of P53 increases transcription of MDM2 that leads to P53 degradation following its ubiquitination [23]. It has been demonstrated that over expressed MDM2 protein reduces anti tumor activity of P53 through binding to this gene [24] and has a key role in resistance to cancer treatment by inactivating this tumor suppressor [12, 13]. Figure 4 illustrates the correlation between P53 and MDM2.

Figure 4. Relations between MDM2 and P53 ^[1]

P53 integrates the cellular response to deathly stimuli such as hypoxia, radiation, drugs, heat shock and DNA damage. In this process, tetramer of P53 activates and arrests cell cycle in both G1 and G2 phases. This protein is able to increase MDM2 that destroys P53 via polyubiquitination and proteosomal degradation.

1.5 Doxorubicin

The strategy in cancer treatment will be based on several factors including (1) type of cancer (2) the stage of cancer (3) patient's age individual (4) cancer progression (5) tolerance to chemotherapy's side effects in patients. There is a variety of cancer therapy including chemotherapy, biological therapy, radiation therapy and surgery [25]. Chemotherapy is the use of cytotoxic agents to fight and kill the cancer cells. In this method the dividing cancer cells are protected from further reproduction. Chemotherapeutic agents work through various mechanisms to interfere the cell replication cycle. There are cell cycle specific agents that affect on a specific stage of cell cycle DNA synthesis and mitotic inhibitors which are categorized in this group. Other anticancer agents are considered as nonspecific [25].

Doxorubicin is a water-soluble anthracycline [26, 27] chemotherapeutic drug taken from *Streptomyces peucetius* var. *caesius* and used in treatment of variety of cancers especially in breast cancer. The other examples of anthracycline drugs include Epirubicin and Iadarubicin. This group of drugs are used in numerous and different cancer treatment including lung cancer, gastric cancer, bladder cancer, Hodgkin's disease, thyroid cancer ovarian cancer and soft tissue sarcoma. Anthracycline has a long half-life and could be administrated as a weekly or monthly infusion [28]. The structure of Doxorubicin is shown in Figure 5.

Although its role of Doxorubicin is remained still unclear however, it affects on Topoisomerase II after unwinding DNA [29] and prevents double helix formation of

DNA which stops replication. Furthermore, this drug inhibits cancer cells growth and dividing. As an iron-chelator, Doxorubicin binds to DNA and cell membrane and cleaves the cells through producing free radicals [30]. It is shown that Doxorubicin mediates in cell differentiation and apoptosis in diverse tumor cells [31]. Some other roles of Doxorubicin consist of alteration in cell membrane structure [32] as well as intercalation in bases of DNA [33].

Figure 5. Doxorubicin structure ^[41]

The anti cancer activity of this agent occurs during two processes:

After Doxorubicin enters the cell, it is transformed to the form of semiquinone [34]. This reduction form produces hydroxyl radicals resulting in cell death through inducing oxidative damage in the cells [35-37]. The other mostly applied process in DNA targeting

drugs is in the nucleus [38]. Here, Doxorubicin bounds to topoisomerase II and inhibits formation of double strand DNA that leads to cell death [36, 39]. The effect of Anthracyclines on DNA replication is shown in Figure 6.

Following Doxorubicin therapy some side effects are appeared which can be categorized in temporary and lasting side effects.

Temporary side effects are referred to the some general symptoms that occur due to use of most chemotherapeutic drugs and will be disappeared by stopping the treatment. Hair loss, sore mouth, fatigue, gastrointestinal upset, loss of appetite and leukopenia are in this group. The less common but serious side effects with considerable affects on patients even after they cured from cancer are categorized in long term group including permanent menopause, loss of fertility and cardio toxicity that should be considered as major side effects lead to heart failure and death in patients [40].

Figure 6. DNA replication and effect of Anthracyclines on this process ^[42]

This agent inhibits RNA and DNA enzymes that are important in protein synthesis as well as restriction of Topoisomerase activity. Anthracyclines affect on DNA and causes transformation of DNA strand from helix to strand shape which leads to inhibition of DNA replication.

1.6 MCF7 Cell Line

The origin of MCF-7 breast cancer cell line is a 69-year-old Caucasian woman [43]. This tumor cell line expresses estrogen and progesterone receptor as well as epidermal growth factor and E-cadherin receptor. It is able to express vimentin and basic fibroblast growth factor whereas many of the matrix metalloproteases are not produced by this cell line.

In this research, our project includes doing proteomics on two samples: breast cancer plasma samples and breast cancer cell line.

In the first part of our research, we were going to link genomics and proteomics together in order to find out remarkable biomarkers by considering MDM2 mutation whereby help to identify Doxorubicin resistance and body reaction to this treatment in breast cancer patients. For this purpose, we were studying on protein expression in cancer plasma samples with two different MDM2 SNP309 genotypes in comparison with the wild type ones by using proteomics method. The aim is investigation of significant biomarkers to show the difference protein profiling in homozygous and heterozygous mutated gene compared with wild type. Since plasma is considered as a rich source of protein patients plasma samples were used for protein identification.

The second part of project related to do proteomics in order to find out useful biomarkers based on a comparison between treated MCF7 cells with Doxorubicin and untreated cells. By this way we would be able to diagnose whether this kind of therapy has an effective role in fighting against breast cancer cells or not.

2. Literature Review

Breast cancer is known as the most common cancer in women population especially in developed countries. Early detection and effective treatment seems required to reduce the mortality of the disease.

Biomarkers are valuable for early diagnosis as well as monitoring the treatment including the identification of resistance in patients. In the last few years, the advances in proteomics through the development in mass spectrometry have opened a hopeful gate in specific and new protein discovery leads to investigation of biomarkers in variety of disease such as breast cancer [44]. In recent years, proteomics methods such as LC-MS/MS (Liquid Chromatography tandem Mass Spectrometry) have expanded to identify milestone biomarkers in clinical samples. LC-MS/MS has been proven to have high accuracy and high resolution spectra.

In our research, we used two different samples: plasma samples of breast cancer patients undertaking Doxorubicin and MCF7 cells that are relevant to breast cancer. First, we discuss about plasma sample and the relevant experiments have been done and the next part of our discussion will be specified to the cancer cells experiments. In the related part of our project to the plasma samples, we propose to study the correlation of MDM2 mutation and protein expression in two different genotypes including homozygous and heterozygous of breast cancer patients undertaking chemotherapy with Doxorubicin, a topoisomerase II-targeting chemotherapeutic drug, by using LC-MS/MS proteomics approach. The main objective of this project is to find effective expressed biomarkers

which should facilitate the monitoring of Doxorubicin treatment that is the most common and effective drug in breast cancer therapy. It exerts its anti tumor role via several mechanisms such as mediating in DNA unwinding and topoisomerase II activity, DNA bases intercalation, alteration in cell membrane structure and differentiation, apoptosis and cell growth [45].

2.1 Breast Cancer, Plasma and Proteomics

Plasma is considered as a great source of proteins that used in purpose of clinical sample and aims to early diagnosis of several diseases includes breast cancer. Why plasma?

It is easy to take plasma from blood patients and this sample is commonly used in routine clinical tests for disease diagnosis [46, 47]. Until now, just few biomarkers in plasma or serum samples have been discovered in breast cancer diagnosis. Some of them such as CA 15-3 or CEA (Carcinoembryonic Antigen) are most routinely used whereas HER-2 or TPA (Tissue Polypeptide Antigen) are less common used in breast cancer detection [20]. However, the discovery of more significant biomarkers in cancer diagnosis and treatment monitoring seems needed to help early diagnosis, prognosis, monitoring the treatment, detection the response and resistance of patients to drugs and the probability of metastasis.

Regarding to a review on 2005 by Srivastava, S. and his colleagues on the importance of plasma protein profiling in early diagnosis and treatment of breast carcinoma, there are two major proteomics approaches in biomarker discovery: a nondirected (global) and target specific approach [48]. In the global approach that is more suitable for biomarker

identification often two technologies such as MALDI-TOF-MS or SELDI-TOF-MS are used. By using these methods many samples can be analyzed in a short period time. The difference between these methods is in the process before analysis in MS and mass/charge peak generations. In SELDI-TOF-MS, based on hydrophobic/hydrophilic or electrostatic forces peptides bind to a solid support. While unbinding peptides removed from the sample. Despite SELDI, in MALDI-TOF-MS, peptides solid matrix will be bombarded with the laser and make the peptides ionized. In TOF part these charged peptides are separated whereas those with less charge will be removed [47].

In the second method, target specific, we used known targets such as antigens or antibodies to decipher a specific protein. In this approach the most common methods are including Western blot, antibody array and Enzyme Linked Immunosorbent Assay (ELISA). The disadvantages of these methods are being time consuming as well as low throughput. Whilst despite the global approaches, they are applicable in clinical experiments [47].

In Table 1 some pros and cons of two major proteomics methods are shown.

Table 1. Proteomic Approaches Used for Breast Cancer Biomarker Identification in Plasma ^[48]

Approach		Example of method	Drawbacks	Advantages
Target Specific		ELISA, Western, Ab array	Loss of detection in untargeted proteins, unavailability for all Ab	Applicable in clinic High throughput
Global	Unidentified proteins	MALDI-TOF-MS	Difficult validation high abundant proteins be detected rarely applicable in clinic	Applicable in clinic High throughput
	Identified proteins	Tandem Mass	Time consuming fractionation, enrichment and depletion are needed	Successful in protein identification high reproducible unbiased search

ELISA, Enzyme Linked Immunosorbent Assay; MALDI, Matrix-assisted Laser Desorption/Ionization; MS, Mass Spectrometry; TOF, Time-Of-Flight ; Ab, Antibody

Wang and his group worked on proteome profiling in body fluids and used liquid chromatography for protein fractionating before analysis in MS part. Through this way, in a strong cation or anion exchange column peptides will be separated based on their charge and then analyzed in mass part [49]. In this method the number of detected proteins is considerable and significantly more than 2D gel electrophoresis.

Based on a research was done by Gramolini et al. [17] since 1980s to now the most common approach in biomarker discovery is mass spectrometry. They searched many published relevant articles to the protein expression in plasma, tissue and urine samples of different cancers from 2003-2007. In spite of its high application in research experiments and the capacity of identification of thousands proteins in samples this approach was not successful in biomarker discovery in breast cancer field. Although many experiments have done in proteomics and biomarker identification in breast cancer just few of them are applicable in clinical diagnosis [20]. One of the challenges in biomarker discovery of breast cancer in plasma samples is the high abundant proteins with high concentration in plasma including albumin, transferrin, immunoglobulins and α 1-antitripsin [17]. These proteins prevent the detection of low abundant proteins in plasma. In some researches for investigation of biomarkers in breast cancer the scientists have got some down or up regulated proteins in serum or plasma of breast cancer patients while these proteins are considered as high abundant proteins.

Another study was performed by Belluco *et al.* on 2007 aimed in protein profiling in breast cancer serum samples [50]. They used SELDI-TOF (Surface Enhanced Laser Desorption Ionization Time of Flight) method in early stage of breast cancer patients. They found out that low molecular weight (LMW) peptides are associated with high abundant ones such as albumin in serum samples. Therefore, they used SELDI-TOF combined with MS to focus on carrier albumin proteins. Then they added acetonitrile to release low molecular weight proteins and make them available to investigation by mass

spectrometry. Belluco and his colleagues believed that these LMW peptides in serum can be helpful in diagnosis of early stage of breast cancer.

In 2006, Stephane Roche and his colleagues were working on serum protein profiling [51] used the same method was used by Belluco and his group. They depleted high abundant proteins by using chicken antibody against them. It is shown that high abundant proteins inhibit expression of low concentration ones in serum or plasma samples and interfere with biomarker investigation. First of all they removed major serum proteins by using immunoaffinity or immobilized methods followed by 2-D electrophoresis [52, 53]. Some major high abundant serum proteins including albumin, IgA, IgG, IgM, ApoA-I and ApoA-II, Transferrin, α 1-antitrypsin, haptoglobin, Fibrinogen, α 2-macroglobulin were removed by this way [54]. Then they used this serum in SELDI-TOF proteomics profiling to identify low concentration peptides in serum.

α 1-acid glycoprotein concentration in patients with three different cancers included breast, lung and ovary carcinoma was studied by Jean-Claude Duche and his research team [55]. This protein as an acute phase protein that will be increased in even up to 5 fold when body faces to acute phase conditions such as stress, pregnancy, inflammation, infections and of course cancers. The concentration of this protein in normal and healthy people is between 0.4-1.1 gr/L [56]. It was shown that among these three groups, breast and lung cancer patients showed an increase around 2.5 times in α 1-acid glycoprotein concentration in plasma. All these patients were undertaking chemotherapy with some anticancer drugs including Doxorubicin. They used immunonephelometric methods for α 1-acid glycoprotein measurement. Although α 1-acid glycoprotein concentration was

increased in ovary plasma, it was not significantly increased as well as two other group cancer patients. Another study was done in 2000 by Mastroianni et al. related to protein expression in plasma of breast cancer patients under Tamoxifen therapy [57]. Tamoxifen was introduced in 1970 decade. It is an antiestrogen drug which is commonly used in advanced breast cancer [58]. They measured Cholesterol (total, low and high density cholesterol), triglyceride and Apolipoprotein A-I in three groups of females; patients under taking Tamoxifen (T^+), patients without Tamoxifen (T^-) therapy and healthy women (control). Based on their experiments total and low density cholesterol (LDL) were dramatically decreased in (T^+) group in comparison with two rest groups, (T^-) and control. From the other hand, the mean concentration of HDL (high density cholesterol) showed a significant increase in (T^+) group with respect to two other ones whereas no considerable differences in the amount of triglyceride and Apo A-I was illustrated in (T^+) comparing with (T^-) and control samples.

Another research was done in 2006 by Huanget et al. [59]. They found three up-regulated proteins: proapolipoprotein A1, transferin and hemoglobin while apolipoprotein A1, apolipoprotein C-III and haptoglobin $\alpha 2$ were identified in lower expression in breast cancer serum. In this study, they used combination of 2-D gel electrophoresis and MALDI-TOF/TOF method [60].

2.2 Breast Cancer Susceptibility, MDM2 and Topoisomerase Resistance

MDM2 (Murine Double Minute 2) plays an important role in liability to cancer and resistance to cancer treatment by inactivating P53, a tumor suppressor [61, 62]. It is therefore a negative P53 gene regulator [31]. Mutation in 309th nucleotide in promoter of this gene affects the response to topoisomerase II-targeting agents such as Doxorubicin. This mutation is a substitution of G instead of T that can be homozygous (GG) or heterozygous (TG) that leads to over expression of MDM2 mRNA [31] and results in binding MDM2 to topoisomerase II. This binding causes reduction of topoisomerase II in the cells. Topoisomerase II is a nuclear enzyme which plays an important role in DNA replication [61] and categorized in two types: topoisomerase I and II. The Type II can be further divided into II α and II β . The expression of topoisomerase II α differs in cell cycle phases (Figure 7). For example, its peak is in G2-M and the lowest level is during G1 and S [61].

Figure 7. Topoisomerase II Expression During Cell Cycle

The expression of Topo II varies in different stages of cell cycle. The expression peak is in G2-M while during G1-S the lowest level is seen.

The function of a significant tumor suppressor, P53, is regulated by MDM2 through different mechanisms: MDM2 prohibits P53 transactivation by binding to it [22]. Moreover MDM2 mediates activation of some important proteins such as P21 in cell cycle. P21 regulates G1 phase in cell cycle process but independent of P53 [63]. Additionally MDM2 impresses on S phase progression of cell cycle by affecting on E2F1, a transcriptional factor, and accelerates its function in contrast to its effect on P53 [64]. MDM2 may influence on resistance or sensitivity of cancer cells to chemotherapeutic

drugs such as Doxorubicin. Preliminary results by other investigators have supported this possibility.

Figure 8. MDM2 Gene with Single Nucleotide Polymorphism ^[31]

A- P53 binding position to MDM2 gene and the occurrence of SNP309 in intron

B- SNP309 is caused by a substitution of Guanine instead of Thymine in 309th nucleotide of MDM2 gene. In this picture wild type and mutated genes are compared

Figure 9. Relation Between MDM2 and P53 ^[65]

The effect of SNP309 on MDM2 is demonstrated. In the presence of SNP309 mutation, SNP309 increases MDM2 level in cells that leads to production of binding site of Sp1 transcription factor in MDM2 promoter. Higher cellular expression of MDM2 diminishes p53 apoptotic activity. In consequence, the presence of G-allele of SNP309 cell cycle arrest and apoptosis processes will be reduced during the stress. By this way, mutated cells will not be removed and cancer arises earlier with high frequencies. Moreover, this mutation in cells causes the resistance to anti-cancer agents.

The relationship between down-regulated topoisomerase II and reduction of sensitivity to VP-16, a topo-targeting agent, was revealed by Chen et al. [45]. Previous studies have shown a reduction of apoptotic response to DNA-damaging drugs such as VP-16 and

gamma irradiation in homozygous cells for MDM2 SNP309 [65]. This is summarized in Figure 8 and 9.

In recent years it has demonstrated that SNP309 in MDM2 causes topoisomerase II degradation that results in resistance to this kind of drugs [61]. It has been demonstrated that homozygous cells for SNP309 had a 10-fold resistance to topoisomerase II-targeting drugs [61]. According to the experiments, nearly 40% and 12-17% of population carry homozygous and heterozygous of this mutated gene respectively [61].

Nayak et al. studied on some various cell lines with wild type (TT) of MDM2 and homo/heterozygous cells for SNP309. These cell lines were taken from different cancers. They studied on the concentration of topoisomerase-targeting drugs such as VP-16 in wild type, homo and heterozygous cancer cells, pursued the response of cancer cells to these drugs and analyzed this aspect with and without regarding to P53. By this way they demonstrated that this response is independent from P53. It is revealed that MDM2 moves between cytoplasm and nucleus [60]. Whereby regulates P53 activation. Consequently, mutation on MDM2 causes inactivation of P53 which results in susceptibility to cancer and even cancer treatment.

In 2006, SELDI-TOF/MS feature was used by Ricolleau and his colleagues [66] to identify important cytosolic proteins expressed in breast cancer patients. They gained good results for prediction of disease prognosis. Their finding is very useful to choose a suitable treatment based on protein profiling.

In this part expressed proteins by plasma proteomics of our samples are briefly explained while detailed results of protein expression in breast cancer patients' plasma samples have been shown in discussion part of this thesis.

Proteins below were significantly expressed (up or down regulation) in treated patients' plasma samples.

Apolipoprotein A1 precursor (APOA1, P02647) is a secreted protein that is released from liver into the plasma. It is also produced in small intestine. Major of HDL cholesterol and chylomicrons are made of Apolipoprotein A1. Moreover, this protein has been detected in the structure of lumen of endoplasmic reticulum and endocytic vesicles. Participation in reverse transport of cholesterol from tissues to the liver is the function of this protein [67]. It promotes cholesterol efflux from tissues and is very important cofactor for LCAT (Lecithin Cholesterol Acyltransferase). APOA1 acts as a binding receptor for beta-amyloids, cholesterol and phospholipids [68, 69]. Figure 10 illustrates APOA1 structure.

Figure 10. Structure of Apolipoprotein A-1

http://www.landesbioscience.com/curie/images/chapters/Akerstrom_vanDijk.jpg

Alpha-2-macroglobulin (A2MG, P01023) has a peptide stretch, called the 'bait region' which contains specific cleavage sites for different proteinases so it is called a proteinase inhibitor. It can inhibit all four classes of proteinases [70]. This protein is also able to inhibit thrombin and plasmin activity and thereby inhibits both coagulation and fibrinolysis processes in the body [71]. Alpha-2-macroglobulin can affect on natural killer cells and Cytotoxic T cells activity to help them as a cytokine neutralizer [72].

Ceruloplasmin (CERU, P00450) is a copper-binding glycoprotein secreted by liver and is released in plasma. Ceruloplasmin oxidizes iron (II) to iron (III) through its ferroxidase activity. It also transports iron across the cell membrane [73]. Ceruloplasmin is an acute phase protein. It is over expressed in pregnancy, inflammation, lymphoma and Alzheimer's disease.

Serotransferrin (TREF, P02787) produced from liver and secreted to the plasma. It has an important role in iron transportation from heme degradation cells to storage and reuse sites. Serum transferrin can stimulate and interfere in cell proliferation [74].

Hemoglobin subunit beta (HBB, P68871) is a major protein in Red Blood Cells and made of two α chains and two β chains in adult hemoglobin. The major role is transporting oxygen from lung to tissue. HBB deficiency has reported in sickle cell anemia, β thalassemia and Heinz body anemia [75].

Clusterin (CLUS, P10909) has a binding ability to the membrane, hydrophobic proteins and cells. Because it can cluster red blood cells called Clusterin [76]. Clusterin is one of

principal proteins in body fluids including plasma, urine and milk as well as semen. This protein interacts with APOA1 [77]. Clusterin is known to be expressed in a variety of tissues and it seems to be able to bind to the membranes and hydrophobic proteins. Although the function has not been known yet well, its association with programmed cell death (Apoptosis), immune response, and complement pathways [78, 79] has been demonstrated. High expression of clusterin occurs in proliferation and apoptosis (programmed cell death in abnormal cells) and some diseases such as myocardial infarction, atherosclerosis, Alzheimer's disease and retinitis.

This protein has an anti-apoptotic activity during tumor genesis and progression stages of some kind of cancers in the cytoplasm. In addition, it mediates in pro-apoptotic activity of Bax protein in the nucleus [80]. Redondo et. al illustrated the over expression of clusterin in the late stages of breast cancer progression. They showed that in breast tumor cells with high expression of clusterin the apoptosis activity will be decreased [81].

Alpha-1-antitrypsin (A1AT, P01009) is a secreted glycoprotein from liver that inhibits trypsin activity in serum. It is also a kind of protease inhibitor with a wide variety of inhibition activity including serine protease inhibition. This enzyme will be increased in acute inflammation to help the cells against inflammatory enzymes such as Elastase. In presence of alpha-1-antitrypsin, Elastase would not be able to break down elastin that leads to severe disorders in lung function called emphysema [82]. A1AT is also able to inhibit plasminogen, trypsin and chymotrypsin activator. The level of A1AT during inflammation is expected to be raised. The structure is shown in Figure 11.

Figure 11. Structure of α -1-antitrypsin

<http://www.cbms.mq.edu.au/~plantserpins/Fig%201%20alpha-1-antitrypsin.jpg>

2.3 Breast Cancer, MCF 7 cells and Doxorubicin

In recent years, research effort has been focused on biomarkers related to breast cancer discovery and treatment monitoring [12, 16, 45, 60] with using proteomics approach. However, most of them utilized 2D electrophoresis that has been proven laborious and less sensitive compared with the LC-MS/MS method that we proposed to use in this project. Since secreted proteins from cancer cells can be considered as a valuable source in biomarker discovery, another part of our project is related to breast cancer cells and protein profiling in treated cells with Doxorubicin.

Mbeunkui and his research team worked on MCF10 protein profiling in order to investigate significant effecting biomarker in aggressive phase of cancer by using LC-

MS/MS approach [16]. They used 4 different MCF10 cell lines including MCF10A, MCF10AT, MCF10 DCIS.com and MCF10CA.cl. D that represent nontumorigenic, premalignant/tumorigenic, tumorigenic/locally invasive and tumorigenic/metastatic, respectively. Following cell culture and harvesting the cells, protein digestion was done to prepare the samples for analysis by LC-MS/MS. Their findings show that in aggressive cell lines, MCF10 DCIS.com and MCF10CA.cl. D, the secretion of α -1-acidchymotrypsin, galectin-3-binding protein, α -1-antitrypsin, cathepsin D and lysyl oxidase is highly increased. Conversely, the abundance of actin and tubulin was low.

On 2001, Mealey et al. [83] studied on the effect of treated MCF7 cells with Doxycycline on resistance to anthracycline agents such as Doxorubicin. It has been determined that treatment with antimicrobial drugs like Doxycycline induces P-glycoprotein (P-gp) expression in cancer cells [84]. P-gp is a protein that acts as a drug efflux pump with lipophilic agents substrates including Anthracyclines [85-87]. However, when cancer cells exposed to these antimicrobial agents P-gp is highly expressed that causes resistance to chemotherapeutic drugs. These findings taken from in vitro experiments while the efforts of chan [88] and his colleagues in 1997 and Nussler research group [89] on osteosarcoma and leukemic patients, respectively demonstrated the role of P-gp high expression in resistance of patients to chemotherapy. This resistance to some lipophilic chemotherapeutic agents such as Anthracyclines leads to treatment failure in most of cancers including breast cancer.

Another study was done by Brown and colleagues to investigate Doxorubicin resistance in MCF7 cells. They turned to use proteolytic ^{18}O labeling. It is considerable to know

that some proteins have *PI* and molecular weight that makes them difficult to identify through 2D gel electrophoresis. In order to eliminate this obstacle Brown et al. applied in proteolytic ^{18}O labeling method. HSP27, Metalloprotein, 40S ribosomal proteins S4 and Thioredoxin were significantly up regulated in resistant MCF7 cells [38]. In 2006, antibody microarray was employed to identify novel biomarkers in Doxorubicin resistant breast cancer cell line as a new and powerful approach [90]. In this research 224 intracellular proteins including cell signaling and apoptotic proteins were studied. It was illustrated that some of these proteins such as Cytokin 18, Cyclin B1 and Cyclin D2 were down regulated in resistant cells. Western blot confirmed micro array results.

The function of some up regulated proteins in our research is explained below:

Keratin typeII cytoskeletal 8 (K2C8, P05787) is a cytoplasmic protein and member of intermediate family [91]. This structural protein is a heterotetramer made of two type I and two type II keratins. Cytokeratin type 8 is associated with Cytokeratin 18. Deficiency in K2C8 causes cryptogenic cirrhosis [92]. Muscle fibers and gingival mucosa contain of this protein.

Glyceraldehyde-3-phosphate dehydrogenase (G3P, P044060) is a metabolic enzyme that produces energy from glucose by breaking down glucose during glycolysis cycle. Moreover, this protein interferes in some other pathways such as Carbohydrate degradation and pyruvate from D-glyceraldehyde 3-phosphate [93]. Figure 12 shows G3P structure.

Additionally, this enzyme participates in other non metabolic cellular process including:

- Transcription activation: It can move from cytosol to nucleus that may approve its role in metabolic and gene transcription activity [94]
- Apoptosis [95]: this protein initiates program cell death through binding to DNA.
- In response to the cell stress NO will nitrosylate Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). S-Nitrosylated GAPDH binds to a ubiquitin protein, Siah1, and moves to the nucleus. In the nucleus the complex of ubiquitin ligase-GAPDH detects proteins aim to degradation which result in apoptosis and cell death [95].

Figure 12. Structure of Glyceraldehyde-3-phosphate dehydrogenase

http://en.wikipedia.org/wiki/File:2-Glyceraldehyde-3-phosphate_dehydrogenase_3GPD_wmpm.png

Peroxiredoxin1 (PRDX1, Q06830) is a metabolic and antioxidant enzyme related to a gene with the same name, Peroxiredoxin (Figure 13). It has an antiviral activity and proliferative effect in progression or development of cancer. It affects on viruses by interfering CD8 T-cells activity [96]. It seems this protein may remove generated peroxides during metabolism in the cell. Peroxiredoxin regulates intracellular concentrations of H₂O₂. Thereby may affect on proliferative process in cells through interfering growth factors signaling pathways of and tumor necrosis factor-alpha [97].

Figure 13. Peroxiredoxin Structure

http://en.wikipedia.org/wiki/File:PBB_Protein_PRDX1_image.jpg

Calreticulin (CALR, P27797) is a calcium binding protein stored in endoplasmic reticulum compartment (Figure 14). This protein found in cytosol and ECM (extracellular matrix) as well as surface of T cells and cytolytic T cells [98].

Calreticulin binds to misfold proteins and prevent them from exporting to the Golgi. Thereby, prevents of protein folding and helps to their degradation. Another protein with the same role is called Calnexin for soluble proteins. These two bind to terminal glucose residues in oligosaccharides in order to break them down. Despite normal situation in protein process that glucose residues will be removed from oligosaccharides during N-

linked glycosylation, Calreticulin and Calnexin bind to these abnormal proteins and prohibit continuing process by preventing the immigration of misfolded proteins to the Golgi apparatus and steer them to degradation pathway [99].

Figure 14. Calreticulin Structure

http://en.wikipedia.org/wiki/File:PBB_Protein_CALR_image.jpg

The presence of Calreticulin propounds the role of this chaperon protein in regulating the transcription process.

Here, most significant down regulated protein in LC-MS/MS analysis of our project based on $PVal < 0.05$ and fold difference less than 0.9 is elucidated. More details of this part can be found in Result and Discussion part.

Heat shock protein HSP 90 (HSP90) is a highly abundant cellular protein [100] (Figure 15). This protein with 90 kDa molecular size is relevant to the chaperon family and a member of heat shock proteins that are secreted during stress [100]. HSP family has a significant role in cell protection during stress resulted from raised temperature. In normal condition its concentration is 1-2% of total proteins whereas this amount will be elevated to 4-6% when cell is heated [101].

This protein has an important role in tumor repression as well as stability, assembling and folding of some proteins such as AKT [102]. Moreover, it affects on cell signaling and protein degradation in unstressed cells.

Figure 15. Heat Shock Protein 90 Structure^[103]

Three functional domains have been known in HSP90 structure such as protein binding, dimerizing and ATP binding domains [104]. ATP binding domain with high affinity to bind with ATP is located near N-terminus. Thereby, HSP90 would be able to break down

ATP in the presence of substrate. It has shown that some antitumor agents target this part of HSP90. However, ATP binding site could be an important part of study in anti cancer drug targeting fields [105].

Protein binding which is located towards C-terminus of HSP90 plays roles in protein folding and clump into proteins. This ability of HSP90 helps to facilitate protein transportation and to inhibit aggregation [106].

3. Significance of Research

Breast cancer is known as one of the important death cause of women in the world. Improved prognosis and effective treatment are needed to reduce the mortality of the disease.

In addition, Singapore has an approximately highest breast cancer incidence in Asia among women population since 1980 up to now. This rate is considered for all three Singaporean races including Indian, Chinese and Malay. Based on PH Tan and her colleagues report the incidence rate of breast cancer is 29.7% of all women cancers in Singapore [107].

Doxorubicin, a topoisomerase II-targeting chemotherapeutic drug has been widely used to treat breast cancer. Effective treatment of breast cancer by Doxorubicin has been complicated by the single nucleotide polymorphism (SNP 309) in MDM 2 gene. The polymorphism is caused by substitution of Guanine over Thymine in its promoter. SNP 309 can be homozygous (GG) or heterozygous (TG) with different frequency in the general population. This polymorphism promotes tumor susceptibility and resistance to topoisomerase II-targeting agents.

We aim to identify plasma proteins which correlate with a particular SNP309 genotype by using LC-MS/MS proteomics approach. The main objective of this research is to find the effective expressed biomarkers which should facilitate the monitoring of Doxorubicin treatment. Study on correlation between secreted biomarkers and MDM2 mutation in

breast cancer patients will reveal the response to Doxorubicin therapy and is a useful way for personalized treatment.

Two different targets were selected in this study: 1) thirty breast cancer patients' on Doxorubicin treatment; 2) breast cancer-derived MCF7 cell line incubated with Doxorubicin. The plasma samples from the patients selected in this study were separated in three groups based on MDM2 single nucleotide polymorphism.

The importance of this research is contemplated from several aspects; first, it could be helpful in studying of treatment efficacy in patients and to clarify whether the therapy is useful or not. It would be useful for physicians in choosing the best treatment strategy. Furthermore, these biomarkers could be used to predict the patient conditions, forecast cancer prognosis, and the fate of therapy regarding to their genotype.

It is to be hoped that proteins identified in our study can be candidate biomarkers in monitoring Doxorubicin therapy in patients.

4. Materials and Methods

4.1 Proteomics

Proteomics is an approach to analyze protein profiling in cell lines, tissues, plasma, body fluids and generally biological systems [18]. This method is applied to discover new drugs, diagnosis the disease in the early stage [108] and patients' response to drug result in access to personalized medicine. Proteomics consists of two stages [109]: first, separation of proteins by digestion. The most common approaches are 2-D electrophoresis and liquid chromatography. Second, protein detection includes of MALDI-TOF-MS that is used with 2-D electrophoresis and ESI or Mass Spectrometry, which usually is combined with liquid chromatography.

4.1.1 Advantages

There have been many different markers such as CA125 for diagnostic and treatment monitoring in clinical experiments. However, it seems this new research era needs more specific biomarkers with high accuracy. In order to reach this aim, proteomics promise a hopeful way. Proteomics can be considered as a reach source for biomarker discovery [13]. This approach could be helpful to gain more information about post-translational changes happen in diseases. Despite proteomics in genomics, gene expression analysis, only transcriptional aspects for thousands genes are identified that it is counted as a weak point in this method. Proteomics technique is more accurate for earlier diagnosis, helps to choose better therapeutic strategies and to discover valuable biomarkers in disease

prognosis. By using proteomics, various proteins are identified while some of them could be very remarkable in disease diagnosis and progress, monitoring the therapy and response to drugs.

4.1.2 Disadvantages

Although proteomics is a useful and new approach in recent years, however some challenges are considerable. One of them is complexity of results are taken from tissue, cell and body fluids that makes it difficult in detection and analysis. In addition, development of bioinformatics and technological approaches are required in this method [110].

There are two kinds of constraints in using proteomics: (a) some of them are related to the protein structure, which is almost observed in proteomics methods, and (b) analytical methods restrictions. Hence, scientists are trying to improve this method for better and more efficient detection of the low-abundance proteins. Moreover, sample preparation for a proteomic analysis seems another withdrawal. It seems difficult and sensitive work because of wide variety of protein molecular sizes, charge, hydrophobicity index, post-translational modifications. From the other hand, mixture of proteins with some macromolecular components and biological characterizations as well as co-factors causes it difficult to follow a protocol for all sample preparations. Thus, an ideal sample preparation protocol should have the capacity of protein insulation in biological samples and removing post-translational modifications and non-specific components such as fatty acids, nucleic acids, plastic polymers and auto-digest peptides [109]. According the

previous studies on proteomics method, low abundance proteins and complexity of them are considered as another challenge. In order to solve these problems, two approaches are proposed [111, 112]:

1- Protein enrichments from huge volumes through using immunoprecipitation, electrophoresis and chromatography.

2- Protein isolation from a mixture into organelle and sub cellular fragment

4.1.3 Most common proteomics features

4.1.3.1 2-D electrophoresis

separates molecules based on their size or isoelectric points via an immobilized PH gradient that is performed by a solution or gel [109].

2DE is able to detect high abundance proteins. However, since most regulator proteins are found in low concentration, this method cannot be responsible in this purpose [113]. This method reveals up/down regulated proteins easily and illustrates highly complex mixtures. Moreover it is an available approach with accessible software while is not a suitable method for extreme molecular weight. The dynamic range is limited. 2DE is considered as a time consuming tool because some steps in sample preparation like staining, destaining and extraction are required. Identification of hydrophobic proteins is another difficulty of 2DE technique.

4.1.3.2 2-D liquid chromatography tandem mass spectrometry

LC-MS/MS isolates peptides based on their charge, size, affinity and hydrophobicities. This approach is able to detect low expressed proteins with various hydrophobicities. Furthermore, it provides a reliable detection via MS/MS automated experiments. Searching on line database is easy and fast through on line data. Besides these advantages there are some drawbacks: for example quantification of protein and posttranslational modified protein cannot be performed efficiently [109]. Chromatographic methods have particular limitation in enrichment of low-abundant proteins. Using this feature leads to enrich both low and high abundant proteins [109] for solving this problem, using affinity chromatography and specific antibodies seem useful. Consequently, the complexity of the protein mixtures will be decreased. It has been shown that techniques based on liquid chromatography reduce signal suppression. As peptide fractionation occurs before mass spectrometry analysis, this technique is considered as a suitable approaches in any given peptide analysis [109].

LC-MS/MS a quantitative proteomics analysis:

Mass spectrometry is a feature to detect proteins as biomarkers [114]. Quantitative protein/peptide analysis in this approach is performed by labeling the peptides and proteins via using isotopes [110]. Currently the most notable aim of proteomics is reach to high accuracy, specificity and reducing the limitations in mass of proteins. This aim has been provided by using tandem mass spectrometry (MS/MS) [115]. Identification of protein in huge of protein mixtures has been performed by using multidimensional chromatography before MS analysis. In this technique, first complex tryptic digests are

separate into several fragments. Then each of these fragments will be more separated using another chromatographic method. Existence of a gradient of salt concentration (KCl) is useful for eluting various peptide fragments from SCX column into Reverse Phase column. Additionally, direct electrospray from nano reverse phase column into the mass spectrometer leads to high sensitivity and automation [115].

LC-MS system is included four parts [113]:

1. Columns: Chromatography columns are designed to separate peptides based on their physiochemical characterization.

This sample introduction is a kind of direct infusion. Two columns SCX and reverse phase column are in the LC-MS/MS analyzer.

Generally, there are three methods for introducing the samples to the ionization source:

A. direct infusion, in LC and GC method

B. Ionization

C. Direct insertion, an insertion plate/probe is used. This is the simplest way to introduce sample into a tool.

2. Ionization source: The eluted peptides are transformed to the gas phase through an ionization source. There are different ionization sources. In LC-MS/MS the ionization source is ESI that is based on evaporation of charged peptide as droplets.

3. Mass analyzer: Two mass analyzers separate ions according on their ratio of mass to charge. The first one is Quadrupole that scans radio frequency and the second is Time of Flight is collaborated with ion's m/z directly. The complex of these two makes machine more sensitive and accurate coupling with mass spectrometry measurement.

4. Detector: Released ion reaches to a detector to identify. In this part ion energy is converted to the electrical signals and transmitted to the computer.

The LC-MS/MS machine is combined of Quad-TOF mass spectrometer for identification and a liquid chromatography system that provides a high accuracy of mass to charge ratio of larger proteins in proteomics analysis. The previous features like GC/MS could only detect the small proteins with molecular weight less than 1,000 Da. whereas by this system there is no limitation for proteins identification. On the other hand, by using LC-MS/MS we can identify a greater variety of proteins with different polarity and molecular weight compared with GC/MS. LC-MS/MS has a high flow rate that leads to high sensitivity of this method.

4.1.4 Sample Running ^[115]

The machine is combined of four parts as mentioned in previous part.

- The first part: Liquid Chromatography (LC) is combined of columns and pumps.

Columns: four columns including one SCX column, one TRAP column, two TRAP columns and Analytical column.

Pumps: Isocratic pump and nano pump

After preparing, the samples mentioned in previous part, mixture of prepared control and patients' samples were transferred to a defined well in the machine. The first step is loading 3 μ l of mixed peptide into the Polysophoethyl A strong cation exchange column, SCX, (0.32 x 50 mm, 5 μ m). In order to elute the retained peptide from SCX column, buffer D contains an increasing concentration of KCl solution 10, 20, 30, 40, 50, 60, 80, 100, 300 and 500 mM is injected in sequence. There are two runs in this step:

In the first run [115] unbounded peptides to SCX column that are trapped on the ZORBOX 300SB-C18 enrichment column I (0.3 x 5 mm, 5 μ m) are removed. Then in order to remove any excess reagent buffer A (5% acetonitrile, 0.1 % formic acid) is used during 100 min with 0.005 ml/ min flow rate. While the other column, ZORBOX 300SB-C18 enrichment column II, is connected to the solvent path of nanopump. Simultaneously, peptides bind to ZORBOX 300SB-C18 enrichment column II and are eluted with buffer B (0.1 % formic acid) and buffer C (95% acetonitrile, 0.1 % formic acid) at 500 nl/min flow rate. In analytical ZORBOX 300SB-C18 reverse phase column (75 μ m x 50 mm, 3.5 μ m) further separation is performed and illustrated in Figure 16a.

Figure 16a First Run in 2D Nano L^[115]

Detail explanation can be found in page 48

In the second run, 5 μ l of 10 mM KCl solution is injected to SCX column to wash out remained peptides to column II. In order to remove the extra reagents, buffer A is loaded for 100 min with the same flow ratio. Now column I containing unbounded peptides in run 1 is switched to the solvent path in nanopump. Peptides are washed out with buffer B (0.1 % formic acid) and buffer C (95% acetonitrile, 0.1 % formic acid) via starting a nano flow gradient from 5% of buffer C which its concentration is increased to 80% over 100 min at 500 nl/ min flow rate. This increasing acetonitrile concentration leads to elute the concentrated sample. Additionally, extra separation is performed in analytical

ZORBAX 300SB-C18 reversed phase column (75 μm x 50 mm, 3.5 μm) and illustrated in Figure 16b. A total of 11 runs are performed to complete one experiment.

Figure 16b. Second Run in 2D Nano LC^[115]

Details were explained in page 50

In the second part, Ionization source, an Electro Spray Ionization (ESI) produces charges for the peptides and sends these charged molecules to the mass analyzer.

In mass analyzer that is combined of Quadrupole and time of flight (Quad-TOF) parts, peptides are detected based on the ratio of mass to charge and compared with our control.

The fourth and last part in LC-MS/MS machine is Detector. In this part, each ion has a current signal. These signals are appeared in monitor of computer and show related peaks.

4.1.5 Data Analysis ^[115]

Data analysis in LC-MS/MS is performed through accurate software named ProteinPilot 2.0 (Applied Biosystems, software Revision 50861). Proteins and peptides are carefully identified in the Protein ID of Protein Pilot software. This is a kind of relative quantitaion. The database for every spectrum of MS/MS is searched for rattus norvegicus species against the uniprot_sprot_20070123. This software works based on following principal criteria:

- fixed modification of methylmethanethiosulfate labeled cystein
- variable iTRAQ modification of tyrosine
- fixed iTRAQ modification of N-terminal and lysine free amine
- side reaction of residues of serine and threonine with iTRAQ solution

Other factors including the accuracy in precursor ion mass and fragment ion mass and tryptic cleavage specificity are considered of protein pilot. Respects to above factors, lack of free amine modification of lysine in iTRAQ or free amine modification N-terminal will not be mediated in protein ratios calculation.

Each detected protein is considered as a biomarker and compared with proteins on Swissprot site that is a well known and reach protein database. Proteins will be accepted on the basis of ProtScore more than 2.0 that give the confidence value of 99%. Protein identification is based on single discarded unique peptides. By labeling control and each sample, each peptide is separated based on its mass and in mass spectrometer label will be detached and released as a single ion that is charged 114.1, 115.1 and 116.1 respectively. Now each sample is shown as a part of peak and displays the abundance of the peptide. Relative protein quantification in use of iTRAQ kit is achieved on MS/MS scans. The ratio of the parts in the peaks of 114, 115 and 116 Da is related to iTRAQ reagents.

By dividing the peaks area at 115.1 and 116.1 m/z to observed area at 114.1 (control), the amount of each peptide in specific sample is estimated. Then overlapping isotopic proteins will be emended whereby the amount of special peptides is calculated.

Error Factor (EF) is a statistic factor shows the errors in ratios and has 95% confidence interval. $95\% \text{ confidence interval} = (\text{ratio} \times \text{EF}) - (\text{ratio}/\text{EF})$. When a protein is considered for more statistical analysis this criteria is required. Two or more unique peptides with high confidence (>95%) must be detected while *P* value is considered to be less than 0.05 and fold difference should be more than 1.06.

Unused protein refers a to protein Pilot measurement of detected protein confidence that is taken to all peptides in a protein, except any peptide with high-ranking protein.

CVpercentage is sequence coverage that is measured by dividing the number of observed amino acids by length of amino acids in a particular protein.

LC-MS/MS coupled with iTRAQ can analyze four samples simultaneously with a same kit.

4.2 Cell Culture and Drug Incubation

MCF-7 cell line was a generous gift of Dr. Koh Cheng Gee's laboratory, School of Biological Science in Nanyang Technological University and maintained in Minimum Essential Medium (MEM) containing 10% Fetal Bovine Serum (FBS), antibiotic-antimycotic (10,000 units/ml Penicillin G sodium and 10 µg/ml Streptomycin sulphate as antibiotics and 25 µg/ml of Amphotericin as antimycotic) were products of GIBCO (Grand Island, NY, USA) at 37°C and 5% CO₂. When the cells reached to 60% confluence they were incubated with 0.1 µM Doxorubicin, was purchased from SIGMA-ALDRICH, for 48 h in the absence of bovine serum. Then trypsin used to harvest both groups of treated and untreated MCF7 cells. Then, cells were stored in liquid nitrogen for further processing.

4.2.1 MTT Assay

MTT reduction test was applied to find out the toxic concentration of drug. We cultured the cells in 96-wells plate and Doxorubicin hydrochloride was prepared with different dilution including 10, 5, 1, 0.5, 0.1 and 0.05 µM. After an overnight chemical exposure in 37°C and 5% CO₂, we removed the medium and incubated the cells with 5 mg/ml dissolved MTT in PBS for 3 h. 100 µl DMSO was added and the plate kept at room

temperature in order to solve the formazan precipitate. By pipetting each well formazan salts were completely solved and the purple dots disappeared. The plates were read on a plate reader (Benchmark plus) using 550 nm as test wavelength and 630 nm as the reference wavelength. A percentage of corresponding value in this method demonstrates the cell viability and the best choice of drug concentration for our MCF7 cells.

4.2.2 Cell Culture

MCF7 cells were taken from liquid nitrogen and melted immediately in water bath (37°C). Then they cultured in...flasks containing MEM medium with 10% FBS and 1% antibiotics and maintained at 37°C with 5% CO₂ and 95% air. Every day the medium was removed and substituted with the fresh one until reaching 60% cell confluence. The cells were harvested by using trypsin and lysed to get intracellular proteins.

4.2.3 Cell Treatment with Doxorubicin

Following determination the best and effective concentration of drug for MCF7 cell treatment through MTT Assay we incubated our cells with 0.1 µM Doxorubicin for 24 hours and harvested them by trypsinization. The extracted protein was prepared for LC-MS/MS running.

4.3 Cell Lysis and Protein Extraction

Harvested calls were lysied by using lysis buffer that was prepared from 4% (w/v) CHAPS, 0.05% SDS (w/v) and 150 μ l of 8 M urea on ice while regularly was vortexed for 20 min. we centrifuged protein at 15000 g at 4°C for one hour. Supernatant was eliminated and precipitate was collected [33].

4.4 Protein Quantification

In order to protein quantification in both plasma samples and cell line we used 2-D Quant Kit from GE Healthcare Company. Plasma samples were used directly in this quantification method whereas in cell lines the first step is protein extraction which mentioned in previous part. By using 6 different concentration of BSA as controls based on kit protocol a standard curve was drawn by a soft ware, Microsoft Origin 6, and the final used volume for our cell line proteins and plasma samples were determined. The next step is sample preparation that in our projects includes plasma and cell line with slight different details.

4.5 Sample Preparing

Plasma was collected intravenously from breast cancer patients. These patients were under taking Doxorubicin and Cyclophosphamide in National Cancer Center of

Singapore. The blood was collected in 2 mL vacutainers coated with 3.6 mg K2 EDTA as anticoagulant then by centrifuging at 2000 g, plasma was separated and stored in -80° C.

4.5.1 Plasma Preparation for LC-MS/MS Analysis

10 µl of each patient's plasma (10 µg/µl concentration*) was used and followed the iTRAQ protocol in order to prepare samples for LC-MS/MS.

In every run, three samples comparing one control can be detected. I had thirty samples and one control. Our control had been taken from a 43-year-old healthy woman.

First, 50 µl of Acetone was added to 10 µl of plasma patient and remained at -20 ° C for 2 hours. Then I centrifuged it in 12900 rpm for 5 min, removed supernatant, and kept precipitant at room temperature for 5 minutes. After that, 20 µl dissolution buffer and 1 µl denaturant were added to the precipitant. After adding 2 µl reducing reagent to this solution, did vortex and spin and incubated 60 minutes at 60° C. In order to block cystine group from the peptide 1 µl of Cystine blocking reagent was used and after vortex and spin then incubated at room temperature for 10 min. Now it is the time of adding trypsin for digestion the proteins. First, we poured 80 µl of trypsin resuspension buffer to the tube contained snow-flaked trypsin at the bottom. Then, 20 µl from this tube was added to each tube containing isotopes 114, 115 and 116 incubated at 37 ° C for 12-16 hrs or 2 hrs in 50 ° C. Then each tube was spined for a few seconds and 70 µl of ethanol was appended.

The important thing should be paid attention is memorizing that each tube is consist of which sample. After spinning and vortex keep them in room temperature for an hour then transferred all tube content in a whole tube and run it in LC-MS/MS machine.

* For plasma concentration we used 2D Quant Kit and followed its protocol

4.5.2 MCF7 Protein Preparation for LC-MS/MS Analysis

Based on protein quantification was done by 2-D Quant Kit 37 and 47 μg of extracted protein from MCF7cells (un-treated and treated with 0.1 μM Doxorubicin, respectively) were mixed with 5 times (150) the sample volume of cold acetone (-20°C) to participate the proteins and kept in -20°C for two hours. The rest preparing process is similar to plasma preparation. In labeling stage we used 114 for untreated cells as a control and 115 for treated cells. The other steps are completely the same with plasma samples.

5. Results and Discussion

5.1 Plasma samples

We used plasma samples of breast cancer patients undertaking Doxorubicin. Plasma is considered as a great source of proteins that used in purpose of clinical sample and aims to early diagnosis of several diseases includes breast cancer. Since both plasma and serum are easy in taking from blood patients and commonly used in routine clinical tests for disease diagnosis [46, 47] we used breast cancer patients' plasma sample in our study. Until now, just few biomarkers including estrogen receptor, HER2-neu and progesterone receptor have been discovered in breast cancer diagnosis. However, more significant biomarkers in cancer diagnosis and treatment monitoring seem needed to be identified.

In this project, we proposed to study the correlation of MDM2 mutation response of breast cancer patients to Doxorubicin, a topoisomerase II-targeting chemotherapeutic drug, by using LC-MS/MS proteomics approach.

The main objective of this project was to find the effective expressed biomarkers which should facilitate the monitoring of Doxorubicin treatment in order to avoid of unnecessary and harmful chemotherapy.

We studied on 30 female patients (29–70 years of age, mean 42.3 years) that were taking chemotherapy with Doxorubicin. They were selected from Chinese (25), Indians (1),

Malay (2), Tai (1) and Pilipino (1) races. Sixteen of these patients were in premenopausal stage and fourteen were menopause.

As mentioned, our patients were at the beginning of chemotherapy and treatment. Inclusion criteria required patients to have adequate bone marrow (absolute neutrophil count > 1 500/ μ l, platelet count > 100 000/ μ l), hepatic (AST and ALT levels $\leq$ 2.5 and total bilirubin < 2.0 times upper limit of normal) and renal functions (serum creatinine < 140 mmol/L) documented at the time of enrollment. Patients with serious co-morbidities including poorly controlled diabetes mellitus, ischemic heart disease, uncontrolled hypertension, active infection or performance status score of > 2 on the ECOG (Eastern Cooperative Oncology Group) scale were excluded from the study. Pregnancy and use of growth factors during the cycle of chemotherapy were also considered as criteria for exclusion.

No forms of endocrine therapy, immunotherapy, or biologic response modifiers were allowed during the period of chemotherapy.

Murine double minute 2 mutation has been studied in this population and it was found that 50% had heterozygous genotype (TG), 26.67 homozygous and 23.33% wild type. All the information of patients was shown in Table 1. Three times running proteomics were done in 15 heterozygous and 8 homozygous SNP309 plasma samples of breast cancer patients. The results are summarized in Tables below based on *P* value less than 0.05 and 95% confidence value.

Table 2. Summary of Patients Information: Patient ID, Race, Age, Age of menopausal and MDM2 genotype.

No	Patient ID	Gender	Ethnicity	Age	Age at menopause	Genotype
1	YLL029	F	CHINESE	29	NA*	TG
2	LCH007	F	CHINESE	33	NA	GG
3	TLH105	F	CHINESE	33	NA	TT
4	CAS053	F	FILIPINO	36	NA	TT
5	SVMP134	F	INDIAN	38	37	GG
6	ZRL152	F	CHINESE	38	NA	TG
7	CSY017	F	CHINESE	39	38	TG
8	LSN082	F	CHINESE	41	NA	TG
9	YMC044	F	CHINESE	42	NA	TG
10	LST104	F	CHINESE	42	NA	TG
11	CLG100	F	CHINESE	50	50	TT
12	ELSM39	F	CHINESE	50	44	GG
13	LGK008	F	CHINESE	45	43	TG
14	LMW99	F	CHINESE	30	NA	TG
15	ATWN025	F	CHINESE	46	NA	GG
16	ML050	F	CHINESE	46	44	GG
17	WLH101	F	CHINESE	46	NA	TT
18	KT141	F	THAI	46	NA	GG
19	LKH018	F	CHINESE	47	45	TG
20	KML094	F	CHINESE	48	47	TG
21	MK065	F	CHINESE	70	47	TG
22	HKW158	F	CHINESE	30	NA	GG
23	TKK086	F	CHINESE	35	NA	TG
24	LKP022	F	CHINESE	41	40	TT
25	TGC131	F	CHINESE	43	41	TG
26	MBA138	F	MALAY	43	NA	TG
27	CAN035	F	CHINESE	46	45	GG
28	TML143	F	CHINESE	47	44	TT
29	SSN120	F	CHINESE	45	NA	TG
30	SBS052	F	MALAY	43	42	TT

*F= Female *NA= premenopausal*

Plasma was collected intravenously from breast cancer women in National Cancer Center of Singapore. The blood was collected in 2 mL vacutainers coated with 3.6 mg K2 EDTA as anticoagulant then by centrifuging at 2000 g, plasma was separated and stored at -80°C.

After preparing the samples for running in LC-MS/MS machine as explained in methodology part, we used isotope 114 for TT genotype as control whereas 115 and 116 were applied for plasma samples of patients with GG and TG, respectively. However, in every run three samples (the first one always was control) were analyzed. Then samples were compared with control and proteins were identified.

The proteins have been up/down regulated and those one with *P* value less than 0.05 selected and considered as significant ones.

In our identification, fold difference (sample/control ratio) has been considered more than 1.06 as up-regulation and less than 1 corresponded down regulation while the value of 1-1.06 meant no significant change in protein expression.

After three runs, the average of 92 proteins detected based on 1.06 fold difference and confidence value of 95%. We categorized patients samples in three groups based on their genotype for SNP309 in MDM 2 gene. The expressed proteins are shown regarding to *P* value less than 0.05. Table 3-6 shows protein expression in both homozygous and heterozygous patients.

Table 3. Down-regulated Proteins in GG Genotype

GG Down Regulation		Protein Name						
Sample		Alpha-2-macroglobulin precursor	Plasma protease C1 inhibitor precursor	Serotransferrin precursor	Alpha-2-HS-glycoprotein precursor	Apo-lipoprotein B100	Beta 2 glycoprotein 1 precursor	Hemoglobin subunit β
LCH 007	Ratio	0.945						0.7291
	PVal	0.0094						0.0038
SVM P134	Ratio	0.7831						0.8023
	PVal	5.3E-80						0.0404
ELS M39	Ratio	0.5136					0.6638	
	PVal	4.2E-06					0.0450	
ATW N025	Ratio	0.6615						0.7218
	PVal	0.0003						0.0467
ML0 50	Ratio	0.7985	0.8016		0.8434			
	PVal	2.7E-10	0.0002		0.0076			
HK W15 8	Ratio	0.9990		0.8437		0.8478		
	PVal	0.0036		6.4E-08		9.3E-06		
KT1 41	Ratio	0.7299						0.8057
	PVal	0.0001						0.0113
CAN 035	Ratio	0.6487						
	PVal	0.0002						

Homozygous patients for SNP309 in MDM2 showed seven Down-regulated proteins including Alpha-2-macroglobulin precursor, Plasma protease C1 inhibitor precursor, Serotransferrin precursor, Alpha-2-HS-glycoprotein precursor, Apolipoprotein B100 precursor, Beta 2 glycoprotein 1 precursor and Hemoglobin subunit β precursor. Among these seven proteins, **Alpha-2-macroglobulin precursor** was down regulated in all sample patients. (PVal < 0.05 and fold difference < 1)

Table 4. Up-regulated Proteins in GG Genotype

GG Up regulation		Protein Name				
Sample		Alpha-1-antitrypsin	Serotransferrin	Fibrinogen α chain	Angiotensinogen	Ceruloplasmin precursor
LCH007	Ratio	1.2544	1.1639			1.1805
	PVal	1.10E06	2.50E-05			0.0218
SVMP134	Ratio	1.2382	1.0692			1.4764
	PVal	2.14E-11	0.0472			1.0010
ELSM39	Ratio	1.0685	1.5356			
	PVal	0.0002	2.06E-16			
ATWN025	Ratio	1.3422	1.1886			
	PVal	3.88E-18	0.0002			
ML050	Ratio	1.4937		1.1720	1.2494	
	PVal	2.39E-14		0.0003	7.11E-06	
HKW158	Ratio	1.8050	1.2800	1.3416		1.1992
	PVal	9.27E-30	7.65E-11	5.80E-05		0.0118
KT141	Ratio	1.3577				
	PVal	1.26E-10				
CAN035	Ratio	1.3041				
	PVal	0.0213				

Patients with homozygous genotype for SNP309 showed up regulation in Alpha-1-antitrypsin precursor, Serotransferrin precursor, Fibrinogen alpha chain precursor, Angiotensinogen precursor and Ceruloplasmin precursor. Among these detected proteins only **Alpha-1-antitrypsin** was up regulated in all GG samples. (PVal < 0.05 and fold difference >1.06)

Table 5. Down-regulated Proteins in TG Genotype.

TG Down regulation		Protein name		
Sample		Serotransferrin	Clusterin	Apolipoprotein A-1
LSN082	Ratio	0.7288	0.807	0.882
	PVal	9.66E-05	0.0004	0.0006
LMW099	Ratio	0.9168	0.688	0.848
	PVal	0.0214	0.001	1.16E-05
ZRL152	Ratio	0.5959	0.857	
	PVal	1.18E-09	0.049	
MK065	Ratio	0.7591		
	PVal	5.27E-06		
SSN120	Ratio	0.5676		0.940
	PVal	4.36E-09		0.05
TKK086	Ratio	0.5613		
	PVal	2.15E-15		
LGK008	Ratio	0.6491	0.921	
	PVal	8.20E-05	0.051	
KML094	Ratio	0.7313	0.924	
	PVal	3.09E-05	0.006	
TGC131	Ratio	0.7815	0.701	0.881
	PVal	0.0512	1.1E-08	0.014
LST104	Ratio	0.8318	0.971	0.850
	PVal	8.76E-07	0.016	0.050
LKH018	Ratio	0.8173	0.939	0.836
	PVal	0.0018	0.051	1.49E-08
CSY017	Ratio	0.7490		
	PVal	0.0078		
MBA138	Ratio	0.5985		
	PVal	0.0002		
YLL029	Ratio	0.6513	0.857	0.824
	PVal	7.4E-09	1.12E-06	0.016
YMC044	Ratio	0.8254	0.893	0.838
	PVal	0.0013	0.0003	0.005

Heterozygous patients for SNP309 in MDM2 gene showed 3 significant down-regulated proteins including Apolipoprotein A-1 precursor, Serotransferrin and Clusterin (PVal < 0.05 and fold difference <1)

Table 6. Up-regulated Proteins in TG Genotype

TG Up regulation		Protein name			
Sample		Ceruloplasmin precursor	Apolipoprotein A2 precursor	Fibrinogen α chain	Fibrinogen β chain
LSN082	Ratio	1.3835		1.1267	
	PVal	1.81E-24		0.0016	
LMW099	Ratio			1.1539	
	PVal			6.36E-06	
ZRL152	Ratio	1.6705			
	PVal	1.05E-16			
MK065	Ratio				
	PVal				
SSN120	Ratio		1.3535		
	PVal		0.0057		
TKK086	Ratio	1.4895			1.2413
	PVal	3.62E-21			0.0411
LGK008	Ratio	1.3888			
	PVal	4.9E-09			
KML094	Ratio				
	PVal				
TGC131	Ratio	1.1176			
	PVal	0.0491			
LST104	Ratio	1.1680			
	PVal	0.0277			
LKH018	Ratio		1.1498		
	PVal		0.0124		
CSY017	Ratio	1.1122	1.3776	1.1983	1.7122
	PVal	0.0002	0.0151	0.0022	0.0038
MBA138	Ratio	1.1020		1.1547	1.3889
	PVal	0.0073		0.0070	0.0323
YLL029	Ratio				
	PVal				
YMC044	Ratio	1.2163			
	PVal	0.0052			

Heterozygous patients for SNP309 in MDM2 gene showed five up-regulated proteins consist of ceruloplasmin precursor, Apolipoprotein A2 precursor, Fibrinogen α and Fibrinogen β chain precursor. In this group, *Ceruloplasmin* precursor was detected in 60%. (PVal < 0.05 and fold difference >1.06)

Table 7 illustrates the summary of significant up and down regulated proteins (*PVal* < 0.05) based on fold difference more than 1.06 and less than 1, respectively in our two groups of plasma samples with homozygous and heterozygous SNP309 genotype in MDM2 gene.

Table 7. List of Significant Expressed Proteins in Breast Cancer Plasma, regarding SNP309 in MDM2 gene

Genotype for SNP309	Down-regulation		Up-regulation	
	Protein Name	Average (±SD)	Protein Name	Average (±SD)
TG	Serotransferrin	0.71 ± 0.10	Ceruloplasmin	1.28± 0.16
	Apolipoprotein A-1	0.85 ± 0.03		
	Clusterin	0.85 ± 0.09		
GG	Alpha-2-macroglobulin	0.75 ± 0.40	Serotransferrin	1.24 ± 0.16
	Hemoglobin Subunit β	0.76 ± 0.04	Alpha-1-antitrypsin	1.35 ± 0.20

Among 92 totally expressed proteins which were taken by running plasma samples in LC-MS/MS machine with considering *PVal* <0.05 only those were selected to more study that expressed more than 1.06 and less than 1 as up and down regulated proteins, accordingly.

In TG group as it is illustrated in Table 7, three proteins including Serotransferrin, Apolipoprotein A-1 and clusterin expressed lower than wild type group. As mentioned in

literature review part, breast cancer patients with this genotype are less resistant to chemotherapeutic drugs from topoisomerase II targeting agents such as Doxorubicin. Down regulation of Clusterin can be considered as a good sign of response to the treatment with Doxorubicin. In 2000, Redondo and his research group worked the relation between breast cancer and Clusterin expression. They showed that in breast tumor cells with high expression of clusterin the apoptosis activity will be decreased. In addition, over expression of this protein can be declared as an indicator of the last stages of progression in breast cancer patients. In consequent, when this protein shows a down regulation in expression we can conclude that apoptosis is under process normally and this demonstrates a good and effective response against tumor cells and thereupon, loss of resistance signs to Doxorubicin.

Further reason for high expression of clusterin in plasma is related to myocardial disorders and atherosclerosis. It is known that Doxorubicin has some side effects including heart damage. High level of clusterin shows myocardial abnormalities so the decrease of clusterin may indicates loss of heart damage resulting from Doxorubicin side effects that is one of significant and harmful effects occurred following Doxorubicin therapy.

This Table shows that APOA1 has been diminished in TG group. From the other hand, clusterin is associated with Apolipoprotein A-1 that is a major compound in HDL cholesterol. It can bind to APOA1 and have some interaction with this protein. In consequent, the abatement in clusterin may results in deduction of APOA1 level in plasma.

The other down regulated protein in plasma of heterozygous patients was Serotransferrin. This protein is one of most important Iron transporters. It has shown that Serum transferrin is able to stimulate and interfere in cell proliferation [74].

Ferritin is a globular protein that binds to Iron molecules which are toxic in free radical form and store Iron in the cells. Ferritin is an acute phase proteins that will be markedly increased during inflammation, carcinoma and infection diseases [116]. In 1976, Jacobs and his colleagues interpreted the relation between high level of serum Ferritin and low saturated transferrin with reaction of reticuloendothelial system to the cancer [117] which may result in inhibition of cell growth [118] and tumor progression because Iron is an important component for DNA synthesis and cell proliferation [119]. In our results decrease in transferrin expression may suggest that our patients with TG genotype are responding to Doxorubicin treatment. Additionally, cell proliferation in these patients is under control that leads to inhibition of disease progression.

In GG group two down and two up regulated proteins were identified. The most significant and discussable one was alpha-1-antitrypsin that is a member of acute phase protein category. This protein will be raised during inflammation or cancer disease in the body. In our GG genotype patients, alpha-1-antitrypsin has been up regulated which show that in these patients the effect of treatment may not be as useful as in TG group. The high level existence of acute phase protein in comparing with our control (wild type, TT)

demonstrates an effective treatment that might be prompted from resistance to Doxorubicin.

It has illustrated that in patients with homozygous SNP 309 in MDM2 gene, the susceptibility to cancer is higher and they are more resistant to topoisomerase II targeting drugs such as Doxorubicin. Additionally, it has been shown that serotransferrin stimulates cell proliferation and has a role in proliferation alteration and based on our experiments in TG relevant results up regulation of serotransferrin may show continuing proliferation in GG group because of their resistance to Doxorubicin and non effective treatment in patients with homozygous genotype in MDM2. Thereby, this protein might be considered as a biomarker in detection of resistant from nonresistant patients to Doxorubicin therapy.

5.2 MCF7 Cell Line

As described in methodology, the second part of our project was running proteomics on MCF7 cell line in order to do protein profiling and investigate specific proteins called biomarker. We cultured MCF7 cells in MEM medium with 10% FBS and 1% antibiotics and maintained at 37°C with 5% CO₂ and 95% air. One group of these cells was incubated with 0.1 µM Doxorubicin for 24 hours. For this purpose when MCF7 cells in MEM medium reached to 60% confluence, we removed medium and added 0.1 µM Doxorubicin diluted with MEM, maintained at 37°C with 5% CO₂ and 95% air for 24 hours (Figure 17). The growth rate in incubated cells with Doxorubicin was less than untreated cells. We incubated MCF7 cells with 0.1 µM Doxorubicin for another 24 hours (Figure 18).

After reaching 60% confluence both treated and untreated cells were harvested by using trypsin and lysed to get intracellular proteins. Figure 17 and 21 show the cell confluence of both treated MCF7 cells treated with 0.1 μ M Doxorubicin and untreated MCF7 cells that used as our control.

Figure 17. Untreated and Treated MCF7 Cells in MEM after 24 hrs incubation

Left: Untreated MCF7 cells after 24 hours incubation at 37°C and 5% CO₂

Right: treated MCF7 cells with 0.1 μM Doxorubicin after 24 hrs incubation at 37°C and % CO₂

Figure 18. Untreated and Treated MCF7 cells in MEM after 48 hrs incubation

Left: Untreated MCF7 cells after 48 hours incubation at 37°C and 5% CO₂

Right: treated MCF7 cells with 0.1 μM Doxorubicin after 48 hrs incubation at 37°C and % CO₂

The next step was proteins quantification done by using 2-D Quant Kit from GE Healthcare Company and Microsoft Origin 6 software. Table 8 shows the protein concentration in both untreated and treated MCF7 cells with Doxorubicin.

Table 8. Protein Concentration in MCF7 cells

	<i>Volume μl</i>	<i>OD</i>	<i>Concentration μg</i>
<i>Control 0</i>	0	0.286	0
<i>Control 20</i>	10	0.269	20
<i>Control 30</i>	15	0.254	30
<i>Control 50</i>	25	0.226	53
<i>Treated MCF7</i>	20	0.230	47.96
<i>Untreated MCF7</i>	20	0.243	37.03

After protein concentration measurement we prepared protein extraction of treated and untreated MCF7 cells for proteomics running. Around 37 and 47 μ g of extracted protein solution from MCF7cells (un-treated and treated with 0.1 μ M Doxorubicin, respectively) were mixed with 5 times (150 μ l) the sample volume of cold acetone (-20° C) to participate the proteins and kept in -20° C for two hours. The rest preparing process was similar to plasma preparation. In labeling stage we used 114 for untreated cells as a control and 115 for treated cells. The other steps were completely the same with plasma samples.

Following 2 times LC-MS/MS running based on accurate software named ProteinPilot 2.0 (Applied Biosystems, software Revision 50861) 252 proteins were detected. Table 9 shows thirteen significant up regulated proteins in treated MCF7 cells comparing with untreated one as a control. The most significant up regulated proteins in our treated cells includes glyceraldehyde-3-dehydrogenase, peroxiredoxin1 and calreticulin which were explained in literature review. The details

Among various roles of GAPDH, its affect on apoptosis will be discussed in this project. Hara and his colleagues showed the important role of GAPDH in apoptosis. During stress conditions in the cell, GAPDH is nitrosylated with NO and binds to Siah1, then moves to the nucleus. In the

nucleus the complex of ubiquitin ligase-GAPDH identifies proteins, initiates apoptosis process and cell death. As a consequence, over expression of GAPDH is suggestive of the ability of cells in inducing apoptosis and defeat tumor. This result may show doxorubicin efficacy in these cells.

The other up regulated protein in our research is Calreticulin. This protein promotes degradation of misfolded proteins. In a research that was done in 2006 by Pugatestch and coworkers the effect of Trastuzumab treatment on breast cancer. Hence, Trastuzumab does not bind to rat cells, their objective cells, B-10 was used instead. They studied on gene expression to identify their relation with cardiotoxocity that is a major side effect of this antitumor drug [120]. After treatment the expression of Calreticulin increased. The role of Calreticulin in inducing apoptosis through activating protein kinase signaling was shown [121].

In our result Calreticulin showed 1.29 up regulation inside the treated cell that may show the reduction of cardio toxicity caused by Doxorubicin therapy. High level of Calreticulin alters in calcium pump function in sarcoplasmic reticulum and calcium release which results in lessening cell contractility [122]. This contractility reduction may be relevant to cardiomyopathy progression in human [120, 123].

After running extracted proteins from treated MCF7 cells and proteomics analysis in compared with untreated cells as control we faced on other significant up regulated protein, Peroxiredoxin. As discussed, this protein may have an influence on proliferative process in cells through altering in growth factors signaling pathways and tumor necrosis factor-alpha, therefore increase of this protein could be considered as an indicator for reduction in cancer progression.

In Table 10, twelve down regulated proteins are shown. Among these twelve down regulated proteins heat shock protein HSP90 could be considered as potential biomarker.

The value of 0.9-1 showed no change in protein expression, the value more than 1 corresponded to up-regulation and the value less than 0.9 meant down-regulation in treated MCF7 cell with Doxorubicin in comparison with untreated cells as control.

**Table 10. Up-regulated Proteins in Treated MCF7 Cells with 0.1 μ M Doxorubicin
Comparing with Untreated Cells Used as Control**

	Protein Name	Accession	Avg. ($\pm$ SD)	PVal
1	Keratin,typeII cytoskeletal 8	P05787 K2C8	1.2 $\pm$ 0.18	<0.05
2	Keratin,typeII cytoskeletal 18	P05783 K2C18	1.15 $\pm$ 0.04	>0.05
3	Elongation factor 1-alpha 1	P681064EF1A1	1.29 $\pm$ 0.22	>0.05
4	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626 ROA2	1.17 $\pm$ 0.08	>0.05
5	Glyceraldehyde-3-phosphate dehydrogenase	P04406G3P	1.19 $\pm$ 0.24	<0.05
6	Peroxiredoxin-1	Q06830PRDX1	1.4 $\pm$ 0.06	<0.05
7	Peroxiredoxin-2	P32119 PRDX2	1.14 $\pm$ 0.22	<0.05
8	Calreticulin precursor	P27797 CARL	1.29 $\pm$ 0.24	<0.05
9	Histone H2A type 1-J	Q99878 H2A1J	1.89 $\pm$ 0.18	>0.05
10	Protein disulfide-isomerase precursor	P07237 DIA1	1.2 $\pm$ 0.22	>0.05
11	Triosephosphate isomerase	P60174 ITPIS	1.18 $\pm$ 0.24	<0.05
12	Pyruvate kinase isozymes M1/M2	P14618 KPYM	1.09 $\pm$ 0.10	>0.05
13	Histone H2A type 2-A (H2A.2)	Q6FI13 H2A2A	1.8 $\pm$ 0.24	>0.05

**Table 11. Down-regulated Proteins in Treated MCF7 Cells with 0.1 μ M Doxorubicin
Comparing with Untreated Cells Used as Control**

	Protein Name	Accession	Avg. ($\pm$ SD)	PVal
1	Heat shock protein HSP 90-alpha	P07900 HS90A	0.87 $\pm$ 0.12	<0.05
2	Heat shock protein HSP 90-beta	P8238 HS90B	0.81 $\pm$ 0.12	<0.05
3	Tubulin alpha-6 chain	Q9BQE3 TBA6	0.79 $\pm$ 0.08	>0.05
4	Nucleoside diphosphate kinase B	P22392 INDKB	0.8 $\pm$ 0.2	<0.05
5	Nucleophosmin	P06748 NPM	0.77 $\pm$ 0.18	<0.05
6	60S ribosomal protein L4	P36587 IPL4	0.77 $\pm$ 0.09	>0.05
7	Nascent polypeptide-associated complex subunit alpha	Q13765 NACA	0.81 $\pm$ 0.06	>0.05
8	Tubulin beta chain	Q9BQE3 TBA6	0.79 $\pm$ 0.08	>0.05
9	Nuclease sensitive element-binding protein 1	Q99878 H2A1J	0.49 $\pm$ 0.2	>0.05
10	Histone H2B type 1-L	P07237 DIA1	0.70 $\pm$ 0.26	<0.05

Here, we discuss about important down regulated protein, HSP90, detected in treated MCF7 cells by LC-MS/MS analysis based on $PVal < 0.05$ and fold difference less than 0.9.

Heat shock protein 90 has an important role in tumor repression as well as stability, assembling and folding of some proteins such as AKT, affects on cell signaling. In tumor cells AKT will be overexpressed [102]. AKT is a kind of cell signaling regulator. This protein is able to defeat G1 and G2 arrest in cell cycle [124]. In addition, it is suggested AKT can elevate proliferation of cells with potential mutation. Chen et. al has shown that this protein may influence on angiogenesis and cancer progression [125]. HSP90 helps to AKT stabilization. By inhibiting HSP90, the function of AKT will be reduced that results in tumor suppression through induction of apoptosis process [102]. Furthermore, vascular endothelial growth factor (VEGF) needs HSP90 for inducing angiogenesis, which is a major process in tumor growth and metastasis [126].

In some papers the correlation of HSP90 and lack of survive in breast cancer patients has been approved. It has shown that HSP90 inhibition affects on some oncogene proteins including BCR-ABL and ERB-B2 and activates apoptosis [127].

In our experiments we identified two isotopes of HSP 90 including HSP 90 alpha and HSP 90 beta.

We observed that in treated MCF7 cells with Doxorubicin in comparison with untreated cells, HSP 90 showed low expression. This result could be considered as a promising sign to confirm successful treatment. It is confirmed that over expression of HSP 90 occurs

during tumor genesis and activates AKT which prevents this protein kinase from motivating apoptosis. Therefore, decrease in HSP90 expression may be related to AKT inhibition and reducing tumor progression. From the other hand, the role of HSP90 in angiogenesis has been demonstrated and hence, down regulation of this protein in treated cells comparing with untreated MCF7 can illustrate the effectiveness of Doxorubicin therapy and useful treatment in this cell line.

6. Conclusion

The result of our plasma samples indicated that Serotransferrin precursor may be considered as a biomarker to indicate resistance or sensitivity to Doxorubicin in breast cancer patients and treatment monitoring. Compared with wild type genotype (TT), this protein was up-regulated in homozygous mutant patients whereas, it showed down-regulation in heterozygous group.

It has been shown that patients with GG mutation for SNP309 in MDM2 gene are more resistant to Topoisomerase II targeting agents including Doxorubicin. In addition, our data indicated that Serotransferrin was significantly over-expressed in GG mutants. These results may be very useful in monitoring patients' response and help physicians in choosing the best treatment so to avoid of ineffective therapy with severe and harmful effects.

LC-MS/MS analysis of MCF7 cells incubated with Doxorubicin revealed three up-regulated proteins include Glyceraldehyde-3-phosphate dehydrogenase, Peroxiredoxin and Calreticulin. One down-regulated protein (heat shock protein 90) was also detected. Based on their involvement cell processes including apoptosis, proliferation, tumor development, these detected proteins may be applicable in studying of patients' response to therapy.

Taken together, the LC-MS/MS based proteomics analysis has been applied to identify biomarkers and map a comprehensive protein profile in cells incubated with Doxorubicin.

This approach has provided us with a better understanding of mechanisms of action of drugs and patients response to the treatment. It is to be hoped that further analysis can lead to better diagnosis, treatment monitoring, prognosis of cancer progress and improvement treatment for the patients [114].

7. Future Prospects

By using LC-MS/MS method in homozygous breast cancer plasma samples two down-regulated proteins, alpha-2-macroglobulin precursor and hemoglobin subunit β precursor, and two up-regulated proteins included Serotransferrin and alpha-1-antitrypsin were detected. In addition, in heterozygous samples Serotransferrin, Apolipoprotein A-1 and clusterin were down-regulated while ceruloplasmin precursor was found to be up-regulated protein.

The importance of these proteins in the body and their relevant role in breast cancer with regarding MDM2 mutation and Doxorubicin treatment was discussed in result part of this thesis.

The other aspect of this project specified to MCF7 cell line and Doxorubicin treatment. In this section the most significant up-regulated proteins were Glyceraldehyde-3-phosphate dehydrogenase, Peroxiredoxin and Calreticulin. Heat shock protein 90 was identified as a down-regulated protein in treated MCF7 cells.

The validation of our findings should include more clinical samples and in vitro analysis. The latter may include Western blot analysis and real time PCR.

Moreover, patients' response to Doxorubicin should be analysed to link their response to the genotype specific proteins identified by LC-MS/MS.

These data could be a promising approach in finding candidate biomarkers that would be applied in clinic to detect resistant or sensitive patients to the treatment.

The validation and further analysis are necessary as this project has been limited by a number of factors, such as number of patients involved, short project time frame, diverse genetic makeup of patients and the lack of comprehensive clinical information of patients

It is to be hoped that data generated from an extended analysis identify candidate biomarkers that would be applied in clinic to detect resistant or sensitive patients to the treatment.

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