Increasing anthracyclines efficacy in Triple Negative Breast Cancer cells by disrupting cells defense mechanism and evaluating Caspase-3 as indicator for anthracyclines hematologic toxicity in breast cancer patients

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**NANYANG
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UNIVERSITY**

SINGAPORE

**Increasing Anthracyclines Efficacy in Triple Negative
Breast Cancer Cells by Disrupting Cells Defense
Mechanism and Evaluating Caspase-3 as Indicator
for Anthracyclines Hematologic Toxicity in Breast
Cancer Patients**

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Breast Cancer Patients**

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A thesis submitted to the Nanyang Technological University in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarized materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

15 Sep 2019

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Date

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Assoc Prof Sierin Lim

Authorship Attribution Statement

This thesis contains material from 1 paper to-be-submitted to Cancer Letters for review where I was the first author.

The results from Chapter 3 and 4 is included in this paper to be submitted as Man Luo, Afu Fu, Na Wei, Kai Song, Sierin Lim and Kathy Qian Luo. G6PD Mediates Metastasis-acquired and Intrinsic Doxorubicin Resistance of Triple Negative Breast Cancer Cells by Maintaining GSH Level.

The contributions of the co-authors are as follows:

- Prof Kathy Qian Luo provided the initial project direction.
- Prof Sierin Lim and Prof Kathy Qian Luo edited the manuscript drafts.
- I prepared the manuscript draft. The manuscript was revised by Dr Fu Afu.
- I co-designed the study with Prof Kathy Qian Luo and performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- All drug treatment experiments, FRET imaging, MTT assay, gene knockdown, Western blot analysis and other functional tests were conducted by me in School of Chemical and Biomedical Engineering.
- Dr Fu Afu established primary and metastatic breast cancer cell lines studied in this research work and assisted in the 2D-DIGE proteomics analysis, including sample preparation.
- Ms Wei Na assisted in the flow cytometry experiments.
- Mr Song Kai assisted in the clinical database search and analysis.

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AUTHOR'S PUBLICATIONS

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Luo M, Tan EY and Luo KQ. Establishment of Caspase-3 Activation as Early Indicator for Hematologic Toxicity of Anticancer Drugs. Singapore Health and Biomedical Congress (SHBC) 2015. (Poster presentation)

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SUMMARY

Chemotherapy is the backbone of systemic treatment for TNBC, among which anthracyclines are important agents and represent the current standard treatment option.

However, treatment failure usually happens due to the drug resistance developed by TNBC cells. Especially when metastasis occurs, the first line anthracycline drug for TNBC treatment, DOX, usually loses its efficacy. The mechanism underlying the metastasis acquired DOX resistance of TNBC cells remains to be elucidated. In the first part of this thesis, two-dimensional difference gel electrophoresis (2D-DIGE) proteomics was applied to compare the protein profiles between an apoptosis sensor-labeled TNBC cell line MDA-MB-231-C3 (231-C3) and its lung metastatic cell line 231-M1 generated in immunodeficient mice that displayed significantly higher cell viability and lower apoptotic rate than 231-C3 cells during DOX treatment *in vitro*. Through analysis by online Panther classification system, one important antioxidant enzyme, glucose-6-phosphate dehydrogenase (G6PD), was hypothesized to correlate to the increase of DOX resistance in metastatic cell line 231-M1. G6PD level was up regulated in metastatic 231-M1 cells compared to 231-C3 cells in proteomics result, which was validated by Western blot analysis. Clinical data analysis showed consistent result that those TNBC patients with relatively higher G6PD expression exhibited lower overall survival rate than lower G6PD expression patients. Either knockdown of G6PD by siRNA or inhibiting its activity with dehydroepiandrosterone (DHEA) successfully reduced cell viability and increased apoptotic rate of the metastatic cell line 231-M1 as well as original TNBC cell lines upon DOX treatment. During DOX treatment, the glutathione (GSH) level was maintained to be higher in 231-M1 cells than 231-C3 cells. After G6PD knockdown, both 231-M1 and 231-C3 cells exhibited lower intracellular GSH level, which coincided with the sensitization of cells towards DOX treatment.

The first part of study in this thesis demonstrated G6PD mediates metastasis acquired as well as intrinsic DOX resistance of TNBC cells and showed G6PD is a potential therapeutic target for sensitizing TNBC towards DOX treatment.

Another challenge that the clinicians are facing during anthracycline administration for breast cancer treatment is the dose limiting drug toxicity, which often leads to treatment delay or even discontinuation and hence the treatment failure. Manifested by dose-dependent neutropenia, hematologic toxicity is the most common acute DOX-associated dose-limiting toxicity, which is closely associated with failure of treatment and an inevitable consequence of non-selective effects of anthracyclines on both cancerous and healthy cells. The second part of this thesis is to develop the caspase-3 activation as an early indicator for hematologic toxicity in cancer patients receiving chemotherapy. Thirty-nine female non-metastatic breast cancer patients were recruited in this project. Elevated plasma caspase-3 levels in cancer patients during chemotherapy course were successfully detected by optimized caspase-3 assay. Moreover, the increased level of plasma caspase-3 activity successfully reflected the severity of hematologic toxicity induced by anti-cancer drugs. Cancer patients in anthracycline-based (AC-T) treatment group displayed higher increase in plasma caspase-3 activity compared with non-anthracycline ones (TC), which agrees with the clinical situation that anthracyclines induce more severe myelosuppression than non-anthracyclines reflected by larger reduction of white blood cell (WBC) count in patients receiving AC-T. Most importantly, in 4 patients from AC-T treatment group, increase of plasma caspase-3 level was observed prior to occurrence of adverse event that WBC count and absolute neutrophil count (ANC) dropped below 50% of baseline level. In conclusion, through this clinical study, plasma caspase-3 level in cancer patients under chemotherapy treatment was proved to be a potential indicator of hematologic toxicity induced by anti-cancer drugs. Caspase-3 level in plasma potentially can serve as a predictor for hematologic adverse event in cancer patients and

hence shows potentiality in guiding dosing schedules of anti-cancer drugs and preventive measures for hematologic adverse event in individual cancer patient.

Overall, through reducing drug resistance developed by TNBC cells toward anthracyclines to improve drug efficacy and also monitoring the hematologic toxicity associated with anthracyclines to promote the safety of drug use, the treatment outcome of anthracycline administration in TNBC patients is expected to be optimized. The treatment failure of anthracycline administration in TNBC patients, especially in metastatic disease, could be reduced in the future clinical practice.

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LIST OF ABBREVIATIONS

TNBC	Triple Negative Breast Cancer
ER	Estrogen Receptor
PR	Progesterone Receptor
HER2	Human Epidermal Growth Factor Receptor 2
MBC	Metastatic Breast Cancer
FRET	Fluorescence Resonance Energy Transfer
DOX	Doxorubicin
ROS	Reactive Oxygen Species
2D-DIGE	Two-dimensional Difference Gel Electrophoresis
G6PD	Glucose-6-phosphate Dehydrogenase
PPP	Pentose Phosphate Pathway
DHEA	Dehydroepiandrosterone
GSH	Glutathione
GSSG	Glutathione Disulfide
WBC	White Blood Cell
ANC	Absolute Neutrophil Count

Chapter 1 Introduction

1.1 Cancer and Breast Cancer

1.1.1. What is cancer?

Cancer is the generic name given to a group of related diseases. There are more than 100 different types of cancer that can develop almost anywhere of human body. The human body is made up of trillions of cells that are basic structural and functional unit of human body. Under normal circumstances, cells grow and divide when they are needed and die when they are old and damaged. When cancer starts, however, genetic changes of cells interfere with this orderly process of cell turnover. Some cells grow and multiply in an uncontrolled manner and may form a growth called tumor [1].

Tumors can be benign or malignant. Benign tumors can only grow in one location and do not spread into other parts of body. Usually, benign tumors can be removed and do not threaten patients' life. However, it could be dangerous when benign brain tumor develops or when tumor grows to a large size. In contrast, malignant tumors can be life-threatening, because they are able to invade and damage the surrounding tissue and when tumor grows, some cells can spread to a distant part that is far from the original location through blood or lymph system.

1.1.2. Types of Cancer

Cancer can be classified into 4 main types based on where the cancer begins. Carcinoma is the most common cancer type. It starts in epithelial cells that line the inner or outer surface of human body and usually forms solid tumor. The most common carcinomas include lung cancer, breast cancer, prostate cancer and colorectal cancer. Sarcoma is a relatively rare type of cancer that arises in the connective tissues. It can affect the bone and soft tissues such as muscle, fat, cartilage, tendons, nerves, deep skin and blood vessels. Leukemia is the cancer of blood cells. It starts in the bone marrow where the blood cells are produced and lead to an uncontrolled generation of abnormal white blood cells. There are 4 main subtypes of leukemia that include acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia and chronic myeloid leukemia. Lymphoma is the cancer that originates in the lymphatic system. It affects lymphocyte, a major type of immune cells being present in lymph nodes, spleen, thymus and bone marrow. The lymphoma can be further categories into Hodgkin lymphoma and non-Hodgkin lymphoma [2].

1.1.3. Cancer stages

There are various cancer staging systems, among which TNM system is the most common and useful staging system for many types of cancer. T represents tumor, providing information of primary tumor size, how deeply the tumor grows into the original organ and whether the tumor cells invade nearby tissue. The larger the number after T (such as T0, T1, T2, T3 and T4), the bigger the tumor or the more it has invaded into the surrounding tissues. N stands for nodes, giving the information

that whether the cancer has spread into the adjacent lymph nodes. The higher the number after N (such as N0, N1, N2 and N3), the greater the cancer cells have spread into nearby lymph nodes. M stands for metastasis, telling whether the cancer cells have been found in distant parts of body. M0 means there is no metastasis can be detected. M1 means the cancer cells have metastasized to distant organs of body [3].

An overall stage, which is numbered from 1 to 4, can be assigned to many types of cancer based on the combination of T, N and M numbers. The higher the number, the larger the tumor size or the more the cancer cells have spread into nearby tissue, lymph nodes or distant organs.

1.1.4. Cancer is a life-threatening disease

Overall, more than 50% of cancer patients can live beyond 5 years. For some types of cancer, the 5-year survival rate can exceed 90%, such as the early-stage colorectal cancer. However, when cancer is diagnosed with an advanced stage or the cancer cells start in or spread to an essential organ, death can be caused. Sometimes, the tumor grows into the digestive system and block or partly block it. Therefore, cancer patients cannot absorb enough nutrients and are unable to acquire enough energy to cope with other problems, such as infection. When the lung is affected by cancer cells, infection can often develop and there might be not enough healthy lung tissues to absorb oxygen. When the bone is damaged by cancer cells, the calcium stored in the bone is likely to release into the bloodstream and thus cause an abnormal increased calcium level in blood, which may lead to unconsciousness of cancer patients. More dangerously, the bone marrow is invaded by cancer cells and thus cannot produce enough blood cells. This may result in

infection and abnormal bleeding of cancer patients due to lack of white blood cells and platelets. When the liver is impaired by cancer cells, the chemical balance in the body is compromised and can be life-threatening. Cancer cells also can damage blood vessels and lead to abnormal bleeding in vital part of body, such as bleeding in brain. One clinical study involving 816 cancer patients reported that the common reason for death of cancer patients were infection, organ failure, infarction and hemorrhage. The most usually happened lethal infections were pneumonia (lung infection), septicemia (blood infection) and peritonitis (peritoneum infection) [4].

1.1.5. Global and local cancer statistics

It is estimated that, as presented in Figure 1-1, in 2018 globally, there will be 18.1 million new diagnosed cancer cases and 9.6 million people will die from cancer [5]. In general population, lung cancer is the most frequently diagnosed cancer, which accounts for 11.6% of the total cancer cases. The second most common cancer is female breast cancer (11.6%), followed by colorectal cancer (10.2%) and prostate cancer (7.1%). In accordance with cancer incidence, lung cancer is also the leading cause of cancer death, which contributes to 18.4% of total cancer deaths. Following lung cancer, the mortality of colorectal cancer, stomach cancer and liver cancer are 9.2%, 8.2% and 8.2% respectively. Among females, however, breast cancer is the most common cancer type and also the leading cause of cancer death. The incidence and mortality of breast cancer in female cancer patients are 24.2% and 15.0% respectively.

Figure 1-1 The global incidence and mortality of common cancers in 2018 for both sexes (upper panel) and females (lower panel) [5]. Reprinted from ref [5] with permission from John Wiley and Sons.

According to the statistical data from National Cancer Centre Singapore, the local cancer cases have been increasing steadily from 2011 to 2015. Despite living in a high level of economic development country with good living environment, 20% to 25% of Singapore residents may develop cancer during their life. For Singapore males, colorectal cancer has the highest incidence, accounting for 17.2% of the male cancer cases. The most commonly diagnosed cancer for Singapore females is still breast cancer that contributes to 29.1% of female cancer cases [6].

1.1.6. Breast Cancer

Because of its considerable incidence rate of 24.2% among all female cancer cases, breast cancer is a huge threat for women all over the world [5]. According to World Cancer Report 2014, 522,000 deaths were caused by breast cancer worldwide in 2012. Due to its high incidence rate and relatively low mortality rate, even when female and male cancer patients are combined for calculation, breast cancer has the highest prevalence among all cancer types, which means in all cancer patients, there are most people diagnosed with breast cancer.

(1) *Breast Cancer Classification*

Breast cancer originates from breast tissue. The symptoms of breast cancer may include a lump or mass formed in the breast, breast swelling, breast shape change, irritation or dimpling of breast skin (sometimes the skin looks like orange peel), nipple turning inward, or fluid shedding from nipple. According to the traditional classification system based on histology, breast cancer can be generally classified into in situ carcinoma and invasive carcinoma. Breast in situ carcinoma can be further categorized into Ductal Carcinoma In Situ (DCIS), in which cancer cells originate from cells lining a breast duct and Lobular Carcinoma In Situ (LCIS), in which cancer cells originate from lobule of breast supplying milk to the ducts. These two types of breast carcinoma are distinguished from each other by cytological features and tumor growth pattern. DCIS is a group of heterogeneous diseases, which are composed of 5 subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid. Similar to breast in situ carcinoma, breast invasive carcinoma can be further classified as 6 histological subtypes: Tubular, Ductal, Invasive Lobular, Infiltrating Ductal, Mucinous (colloid), and Medullary. Among these,

Infiltrating Ductal Carcinoma (IDC) is considered as the most common invasive disease, which contributes to 70-80% of all invasive lesions [7].

With the advancement of high-throughput gene expression analysis, it has been shown that response of breast cancer to treatment is not fully determined by tumor anatomical characteristics but rather intrinsic molecular features [8]. Classification of breast cancer patients based on tumor molecular characteristics provides an increased level of accuracy for disease prognosis and treatment decision-making. In light of gene expression profiling, breast carcinomas are categorized into 5 subtypes with distinct molecular features: Luminal A, Luminal B, HER2 over-expression, Basal and Normal-like. Tumor cells in these subtypes exhibit various expression levels of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2 or ERBB2), cellular proliferation marker KI67 and basal marker and have distinct clinical outcomes.

Luminal A breast cancer is the most common breast cancer subtype followed by Luminal B tumors. Breast cancer patients with luminal subtypes constitute up to 64.3% of all patients. Generally, luminal breast cancers obtain a relatively good prognosis, in which luminal A subtypes have a significantly better prognosis than luminal B tumors. Due to the high expression level of both ER and PR, luminal tumors have a good response to hormone therapy but react poorly towards traditional chemotherapy. Normally, endocrine therapy alone is adequate for luminal A breast cancer treatment, while the combinational therapeutic strategy of hormone therapy and chemotherapy are more beneficial to luminal B tumors due to its high proliferation ability.

Occurrence of HER-2 over-expression breast cancer is not associated with patients' age, race or other known risk factors. Although they are sensitive to chemotherapy based on anthracycline and taxane and have a better pathological complete response compared to luminal breast tumors, this breast cancer subtype exhibits a poor prognosis with a higher early relapse risk. Because of over-expression of HER-2 protein, treatment of this breast cancer subtype benefits from target therapy, such as trastuzumab, one of the anti-HER2 monoclonal antibodies.

The molecular expression profile of basal tumors is similar to the normal breast epithelial cells, which is featured by lacking of ER and PR expression and low expression of HER2. In contrast, these tumor cells are associated with high expression of basal markers as well as proliferation related genes. They are more likely to have TP53 mutation and harbor low BRCA1 expression. This breast cancer subtype attracts a huge amount of research interest, because this disease has an aggressive progression with a rapid growth of tumor and lack systemic targeted therapy. The conventional chemotherapy is the only choice of systemic treatment for this subtype. Basal-like breast cancer has a higher incidence in young-age patient and African-American women and is associated with lower survival rate and higher risk of relapse compared with other subtypes.

(2) Triple-Negative Breast Cancer (TNBC)

Breast cancer cells can be classified according to their receptors that are present on cancer cell surface and in their cytoplasm and nucleus. Breast cancer cells may or may not express three important kinds of receptor: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type-2 (HER2). For the breast cancer cells that have hormone receptors, ER, PR or both, they can be treated with hormone therapy. Hormone therapy (also called anti-

hormone treatment) prevents breast cancer cells from obtaining natural hormones, estrogen and progesterone, which they need to grow. There are a few options for anti-hormone treatment. For ER+ breast cancer, a drug called tamoxifen can be used to block estrogen activity in the body. Alternatively, aromatase inhibitor can be used to prevent the body from producing estrogen for patients going through menopause. Moreover, patients' ovaries which is the organ producing hormones, can be removed by surgery [9]. For HER2+ breast cancer, Trastuzumab, a monoclonal antibody of HER2 which can inhibit cancer cell growth by blocking HER2 activity has been developed as targeted therapy [10].

About 12% to 17% of breast cancer patients are diagnosed to have Triple-Negative Breast Cancer (TNBC) [11]. TNBC is defined as that the cancer cells do not express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type-2 (HER2). Consequently, patients with TNBC cannot be treated with hormone therapy or target therapy aiming to HER2. And chemotherapy also leads to a worse outcome with triple-negative disease than other subtypes of breast cancers. Neoadjuvant studies in which chemotherapy is administrated before surgery suggest that although this treatment is very effective for minority of triple-negative breast cancer patients, the outcome for the majority is relatively poor. Currently, there is no standard chemotherapy strategy for triple-negative breast cancer and the target therapy against this subtype of breast cancer is being intensively researched [12].

1.1.7. TNBC cell lines studied in this project

This project is mainly based on the breast cancer cell line MDA-MB-231. MDA-MB-231 is originated from pleural effusion metastasis of invasive ductal

carcinoma. This breast cancer cell line does not express ER, PR and also without overexpression of HER2. It is a TNBC cell line.

A GFP-based FRET (fluorescence resonance energy transfer) sensor was developed by Prof. Luo Qian Kathy's group to detect caspase-3 activity in living cells. This sensor consists of a CFP (cyan fluorescent protein), a YFP (yellow fluorescent protein) and a special peptide linker containing caspase-3 cleavage site which connects CFP and YFP together [13]. One new stable cell line named MDA-MB-231-C3 (231-C3) was generated by transfection of the plasmid DNA encoding this GFP-based FRET sensor into MDA-MB-231 cells (Figure 1-2).

Figure 1-2 231-C3 cells expressing the GFP-based FRET sensor. Scale bar is 100 μ m [14]. The permission for reprinting from ref [14] is not needed.

Approximately 500,000 of 231-C3 cells were injected into blood circulation system of nude mice through the tail vein. After 45 days of cell injection, the first lung micro-metastasis was found in a mouse with a much bigger tumor size. One stable cell line was generated from this lung micro-metastasis, which is named as 231-M1 (Figure 1-3).

Figure 1-3 The generation process of 231-M1 [14]. The permission for reprinting from ref [14] is not needed.

1.2 Breast Cancer Treatments

1.2.1. Treatment Options for Breast Cancer

Like other cancers, the treatment for breast cancer can be broadly classified into two categories: local treatment and systemic treatment. Local treatment targets tumors grown on a certain part of body, such as surgery and radiation therapy; whereas systemic treatment aims to kill cancer cells spreading all over the body, which includes hormone therapy, targeted therapy and chemotherapy [15].

(1) *Local Treatment*

a. Surgery

Surgery is the most common treatment option for breast cancer patients. Most patients receive some kinds of surgery as part of their treatment strategy. There are different kinds of surgeries:

- **Mastectomy:** this type of surgery is to remove the whole breast tissue. Currently, in most cases, the muscle under breast is not removed, making mastectomy less intrusive.

- Lumpectomy: also known as breast-conserving surgery. Only the tumor and a small part of surrounding healthy breast tissue are removed by this kind of surgery.
- Axillary lymph node dissection: Surgery to remove nearby lymph nodes if cancer cells have been found to spread into lymph nodes close to breast [16].

Based on family history or gene mutation examination, some people at high risk of breast cancer might choose to receive prophylactic mastectomy or prophylactic ovary removal for disease prevention. Surgery is rarely to be used for treating metastatic breast cancer, in which cancer cells have spread to other parts of body [17].

b. Radiation therapy

Radiation for cancer treatment applies the X-ray with high-energy, protons or other particles to destroy the fast-growing cancer cells. The ionization induced by radiation can damage the cellular molecules, such as DNA directly. Meanwhile, by indirect action, free radicals can be produced from ionization or excitation of intracellular water molecules during radiation and cause DNA damage [18]. There are two main types of radiation therapy that can be used for breast cancer treatment:

- External beam radiation: It is the most common radiation therapy used to treat breast cancer, during which a machine outside the body delivers radiation to a localized part of body.
- Internal radiation (brachytherapy): Internal radiation is a less common method to deliver radiation, which is normally used after lumpectomy. To give internal radiation, a device containing radioactive source is put inside body close to the tumor site for a short period.

Radiation therapy is a useful way to reduce the risk of cancer relapse after surgery for primary breast cancer. For metastatic breast cancer, it can also be applied to relieve the symptoms of disease [19].

(2) Systemic Treatment

a. Hormone therapy (endocrine therapy)

Hormones are chemical messengers produced by endocrine glands, delivered through bloodstream and affect the behavior of cells and tissues throughout the body. Hormone-sensitive breast cancer cells express hormone receptors on cell surface, which include estrogen receptor (ER) and progesterone receptor (PR). When hormones (estrogen or progesterone) bind to these receptors expressed on breast cancer membrane, these receptors become activated and promote cell growth and division through changing the expression of certain genes by regulating cellular signaling pathways. To slow or stop the growth of hormone-sensitive breast cancer tumors, hormone therapy is used to decrease the production of hormones by human body or to interfere with function of hormones on breast cancer cells [20].

As one kind of systemic breast cancer therapy, hormone therapy medicines can target the breast cancer cells all over the body. It is frequently applied after surgery to reduce the recurrence risk of early-stage breast cancer (as adjuvant therapy) and also used before surgery to shrink breast tumor for complete tumor removal (as neoadjuvant therapy) [21]. Moreover, hormone therapy can also slow down the progression of advanced-stage or metastatic breast cancer with hormone receptor expression. However, it loses its efficacy against hormone receptor-negative breast cancers.

There are various types of hormone therapy:

- Block the function of ovary: ovarian ablation (also called oophorectomy), in which ovaries are removed surgically or through radiation treatment, is applied to eliminate estrogen production by ovaries permanently. Alternatively, the drugs named gonadotropin-releasing hormone (GnRH) agonists, such as FDA approved leuprolide (Lupron) and goserelin (Zoladex), are also used to suppress ovarian function temporarily through interfering with the signal given by pituitary gland to ovaries for estrogen production.
- Disturbing estrogen production: Aromatase, an enzyme located in cells that produce estrogen, catalyzes the reaction in which testosterone (an androgen) converts to estradiol (an estrogen). So blocking the activity of aromatase by its inhibitors can disturb the production of estrogen. Anastrozole (Arimidex) and letrozole (Femara) are two FDA-approved aromatase inhibitors that are able to inactivate aromatase temporarily. Alternatively, exemestane (Aromasin) is one of aromatase inhibitors with permanent effect.
- Blocking the effects of estrogen: Selective estrogen receptor modulators (SERMs) are a group of drugs that block estrogen receptors on breast cancer cell surface and thus prevent estrogen from binding and activating its receptor. Tamoxifen (Nolvadex) is one example of SERMs with more than 3 decades using history, acting as estrogen antagonists in breast tissue [22]. Toremifene (Fareston), which is another SERM, is used less commonly and only approved for metastatic breast cancer treatment. Unlike tamoxifen and toremifene, Fulvestrant (Faslodex) is a selective estrogen receptor degrader (SERD), exerting its function by inducing estrogen receptor destruction once

it binds to estrogen receptor. It is approved to treat metastatic breast cancer when other hormone therapy drugs lose their efficacy [23].

b. Targeted therapy

The breast cancer cells in some cancer patients overexpress specific receptors on their surface that can activate the downstream signaling pathway and resulting in cancer cell growth and proliferation. Targeted therapy for breast cancer treatment applies certain substances or drugs to interfere with the function of overexpressed receptor and thus lead to tumor regression.

- Trastuzumab (Herceptin) and Pertuzumab (Perjeta) are monoclonal antibodies to target HER2 protein expressed on the surface of cancer cells. They can be used to treat both early-stage and advanced breast cancer when the cancer cells overexpress HER2.
- Ado-trastuzumab emtansine (also known as TDM-1) is composed of HER2 monoclonal antibody that links with a chemotherapeutic agent.
- Lapatinib (Tykerb) and Neratinib (Nerlynx) is two kind of kinase inhibitor
- Palbociclib (Ibrance), ribociclib (Kisqali), and abemaciclib (Verzenio) are inhibitors of cyclin-dependent kinases (CDKs), especially CDK4 and CDK6. They are approved to treat advanced breast cancer that is hormone receptor-positive but HER2-negative.
- Everolimus (Afinitor) are the drugs that target mTOR, a serine/threonine protein kinase in cells that regulate cell growth and proliferation.
- Olaparib (Lynparza) and talazoparib (Talzenna) are inhibitors for PARP protein that is involved in cellular DNA repair and genomic stability. Cancer patients with BRCA gene mutation have compromised DNA repairing

function. Blocking PARP proteins by these two drugs in cells with mutated BRCA genes often leads to cell death.

c. Chemotherapy

Chemotherapy can be used in several situations for breast cancer treatment. In adjuvant chemotherapy settings, chemo drugs are given to patients after surgery of tumor removal, which aims to kill any residual cancer cells to lower the risk of breast cancer recurrence. Chemotherapy can also be used before breast cancer surgery. It is so called neoadjuvant chemotherapy, the advantage of which is shrinking the tumor before removing it, so that less invasive surgery is required. For advanced breast cancer in which cancer cells have spread outside the breast, the chemotherapy is considered as the main treatment in clinical practice [24].

There are various chemotherapeutic drugs that are effective for breast cancer treatment, including Anthracyclines (Doxorubicin, Epirubicin), Taxanes (Paclitaxel, Docetaxel), Platinum agents (Cisplatin, Carboplatin), Vinorelbine, Capecitabine, Gemcitabine, Lxabepilone, Eribulin [24]. It is common that two or more drugs are combined together to treat breast cancer.

1.2.2. Efficacy of Anthracyclines in TNBC Treatment

Because TNBC cells lose target receptors such as ER, PR, HER2, hormonal therapy or targeted therapy is not beneficial to TNBC patients [25]. Chemotherapy is the backbone of systemic treatment for TNBC, among which anthracyclines are important agents and represent the current standard treatment option [26, 27].

(1) Response of TNBC to Anthracycline-containing Chemotherapy Regimen

Several clinical studies supported the advantage of anthracycline-based chemotherapy in TNBC treatment. In one retrospective clinical analysis conducted by Berry and colleagues, the effectiveness of adjuvant chemotherapy was investigated in terms of ER status of breast cancer patients. When the patients were treated with chemotherapy regimen including dose-dense doxorubicin, the ER-negative breast cancer patients displayed decreased risk of recurrence and better overall survival in comparison to patients with ER-positive breast cancer. A 55% relative reduction in recurrence risk was observed in ER-negative patients receiving dose-dense AC-T (doxorubicin, cyclophosphamide and paclitaxel) combinational chemotherapy regimen compared to low-dose AC-T receiving group [28]. In one clinical study involved 1,118 breast cancer patients who were given with neoadjuvant chemotherapy (>80% received anthracycline-based regimen), TNBC patients experienced a significantly improved pathologic complete response (pCR) compared to non-TNBC patients (22% vs. 11%; $P=0.034$) [29]. Rouzier et al. examined the objective response of breast cancer patients with different molecular subtypes to neoadjuvant chemotherapy containing doxorubicin, the highest rates of pCR was observed among TNBC subgroup (45%; CI 24-68%) [30]. This result was further validated in clinical study conducted by Carey et al [31].

Despite the typically better response of TNBC subgroup to preoperative chemotherapy than others and a high pCR rate achieved after anthracycline-contained chemotherapy, the early relapse of TNBC that leads to devastating consequences of cancer patients and the premature development of drug resistance that causes unsuccessful treatment outcome were observed by clinicians [32, 33]. Compared with other types of breast cancer, TNBC has higher rate of relapse with most recurrence occurring in 3 years of diagnosis and early visceral metastasis [32,

34]. Unfortunately, the metastatic breast cancer (MBC) remains almost an incurable disease with a five-year survival probability of only around 25%. More than 90% of MBC patients are suffered from treatment failure due to chemotherapy resistance [27]. Currently, there is no standard chemotherapy regimen for metastatic TNBC [35].

(2) Doxorubicin (DOX)

The trade name of doxorubicin is Adriamycin. It is an effective chemotherapeutic agent belonging to anthracycline antibiotics that is widely used to treat not only breast cancer, but also other kinds of cancers, such as the bladder cancer, lymphoma, acute lymphocytic leukemia and Kaposi's sarcoma. It is usually used in a combinational treatment strategy and given into cancer patient through intravenous injection [36].

Before the discovery of doxorubicin, a similar compound daunorubicin was discovered from a bacterium strain, *Streptomyces peucetius* as an antibiotic and was found to be effective against tumors [37]. Through clinical trial, a serious side effect, lethal cardiac toxicity, is found to be produced by daunorubicin [38]. Through changing the structure of daunorubicin slightly, researchers discovered a new red-colored antibiotic from a mutated strain of *Streptomyces* (Figure 1-4). This new compound was named doxorubicin and it displayed higher therapeutic index compared with daunorubicin, but the fatal cardio toxicity is still associated with doxorubicin [39].

Figure 1-4 Chemical structure of doxorubicin [37]. Reprinted from ref [37] with permission from John Wiley and Sons.

Although DOX has been used for treating a wide range of cancers for more than 4 decades, the exact mechanism of action underlying cancer cell killing effect of DOX remains uncertain and controversial [40].

Inhibition of DNA biosynthesis as anthracycline's mechanism of action was reported by earliest studies. It was described that doxorubicin intercalates into DNA and disrupts DNA repair mediated by topoisomerase-II, and/or inhibits the activity of DNA polymerase [41, 42]. However, some subsequent studies failed to detect the effect of DNA synthesis inhibition exerted by DOX at a relatively low drug concentration [43, 44].

A few years later, another mechanism of action of DOX was proposed that DOX is able to generate free radicals and hence induce intracellular oxidative stress, which attracts interest from a huge population of scientists [45-51]. More specifically, DOX could be oxidized to semiquinone and when semiquinone is converted back to the original molecule, reactive oxygen species (ROS) are generated that lead to increased intracellular oxidative stress [52]. When the oxidative stress overwhelms the antioxidant ability of cancer cells, cell apoptosis is

triggered [53]. In recent study, it was found that ROS generation is one of the mechanisms of DOX-induced cancer cell apoptosis and an antioxidant protein, manganese superoxide dismutase (MnSOD) is involved in the DOX resistance of breast cancer [14].

(3) Mechanism of DOX resistance

There are many mechanisms underlying DOX resistance of cancer cells that have been reported. The up-regulation of multi-drug resistance protein 1 (MDR1; also known as P-glycoprotein) that leads to DOX resistance was reported in earliest studies [54]. Acting as a drug efflux pump, P-glycoprotein (P-gp) that is a 170kd adenosine triphosphate (ATP)-dependent transporter expressed on cell membrane is able to increase DOX efflux and thus reduce intracellular DOX concentration. The other ATP-Binding cassette transporters with the similar function as P-gp that is associated with DOX resistance include multidrug resistance protein 1 (MRP1), canalicular multi-organic anion transporter (CMOAT) and breast cancer resistance protein (BCRP) [55, 56]. In another research work, the DOX-resistant human colon cancer cells that showed increased activity of pentose phosphate pathway (PPP) and glucose-6-phosphate dehydrogenase (G6PD) have lower intracellular DOX accumulation in comparison with DOX-sensitive counterpart. The author concluded that the high intracellular glutathione (GSH) level generated by activated PPP and increased G6PD activity helps cells to extrude DOX out of cells [57]. It was suggested GSH enhancement has effects on multidrug resistance in cancer cell.

It was also reported that down-regulation or mutation of topoisomerase II could lead to DOX resistance. Some studies showed enzyme activity of DNA topoisomerase II and its protein expression level is reduced in DOX-resistant P388 murine leukemia cells relative to drug-sensitive counterpart, which is due to

decreased native mRNA amount and mutation of topoisomerase II allele [58, 59]. It was reported by another study that teniposide (VM-26) resistant human leukemia cell line CCRF-CEM was associated with decreased topoisomerase II catalytic activity level and reduced ability to form covalent protein-DNA complexes that is stabilized by drug. VM-26 is a topoisomerase inhibitor, having similar effect as DOX in terms of interfering topoisomerase-II function [60]. The VM-26 resistance phenotype was correlated with mutated ATP-binding sequence of topoisomerase-II.

Various products of tumor suppressor genes and oncogenes have been indicated to cause DOX resistance. P53 gene specific mutation is related to *de novo* DOX resistance and early disease recurrence in breast cancer patients [61]. Inactivation of protein phosphatase 2A (PP2A), another key tumor suppressor, determines DOX resistance in early stage breast cancer and poor therapeutic outcome [62]. Increased expression of cancerous inhibitor of protein phosphatase 2A (CIP2A) was also showed to be associated with DOX resistance in breast cancer cells [63]. Overexpression of murine double minute 2 (MDM2) was suggested to lead to DOX resistance of breast carcinoma [64].

Development of DOX resistance in cancer cells have also been reported to involve the high expression of cyclooxygenase-2 (COX2) [65], Stat3 activation [66], downregulation of HuR [67], expression of CD44 [68], high expression of aldo-keto reductases (AKRs) [69]. Involvement of MicroRNAs (miRNAs), such as microRNA-125b and microRNA-451, in doxorubicin resistance in human breast cancer cells has also been reported [70, 71].

The tumor microenvironment also was reported to have impact on therapeutic outcome of DOX. In one lymphoma mouse model, the paracrine factors such as IL-

6 and Timp-1 are released by thymus following DOX administration. These pro-survival cytokines promote survival of residual tumor cells after genotoxic chemotherapy and thus leads to eventual tumor relapse [72]. Another study showed IL-8 present in the conditioned medium secreted by mesenchymal stem cells (MSCs) mediate DOX resistance of TNBC cells [73].

Although oxidative stress generation ability of DOX has been intensively studied [45-51], there are few research works that explains DOX resistance of cancer cells in terms of their antioxidant ability. It was suggested that enhanced glutathione peroxidase activity and its increased expression level in DOX-resistant cells protect cells from DOX-induced lipid peroxidation and hence could lead to cell resistance to DOX [74]. A similar finding was reported by another study that glutathione peroxidase detoxifies oxygen radicals induced by DOX and hence contributes to DOX resistance of sarcoma cell lines derived from clinical samples [75]. More directly, load MCF-7 cells with glutathione peroxidase or superoxide dismutase can generate DOX resistance in cells [76].

A previous study conducted in our lab successfully demonstrated that knockdown of MnSOD, a mitochondrial antioxidant protein, significantly sensitized TNBC cells to DOX induced apoptosis by overcoming the protection of cells exerted by MnSOD from mitochondrial damage induced by superoxide (one kind of ROS) [14]. It suggested that antioxidant proteins are able to protect TNBC cells from oxidative stress induced by DOX.

1.2.3. Safety of Anthracyclines in Breast Cancer Treatment

(1) Toxicity Associated with DOX

Adverse drug reactions are often involved in anthracycline administration, among which hematologic toxicity is a major dose limiting toxicity. Myelosuppression may be produced by DOX, which requires careful monitoring. DOX hematologic toxicity is predominantly manifested by dose-dependent leukopenia and/or neutropenia, which is the most common acute DOX-associated dose-limiting toxicity. Leukopenia usually reaches its nadir 10 to 14 days after DOX treatment based on recommended dose schedule. Recovery of leukopenia often occurs by the 21st day of drug infusion. Anemia and thrombocytopenia may also occur. Fever, infection, sepsis, hemorrhage, tissue hypoxia, septic shock, or even death are clinical manifestations of severe myelosuppression caused by DOX [77, 78].

(2) Hematologic Toxicities Induced by Chemotherapy and Its Current Treatments in Clinical Practice

Hematologic toxicity is the most common toxicity caused by anti-cancer chemotherapeutic drugs, often leading to drug dose reduction and discontinuation of drug administration. Since rapidly growing cells are suffered most seriously from majority of chemotherapeutic agents, the bone marrow cells, which are continuously proliferating, are inevitably impaired by these drugs. Myeloid stem cells differentiate into white blood cells (WBCs), red blood cells (RBCs) and platelets, thus suppression of bone marrow activity (myelosuppression) leads to reduced number of these circulating blood cells [79].

Mature neutrophils constitute 50%-70% of circulating WBCs. Their growth and maturation usually need 9-10 days, and they only live for 6-12 hours once they are released to blood circulation. Because neutrophils have short life span and proliferate rapidly, they are very vulnerable to cytotoxicity of anti-cancer drugs. Neutropenia, which is the most common hematologic toxicity, refers to the event

that neutrophil count is less than 500 cells/mm³ or neutrophil count is less than 1000 cells/mm³ with a predicted trend to drop below 500 cells/mm³, often resulting in dose reduction and chemotherapy delay [80, 81]. Toxicity criteria released by National Cancer Institute (NCI) for neutropenia is shown in Table 1. Chemotherapy induced neutropenia is associated with hospitalization for infection treatment, higher economic burden of cancer patients and society, as well as compromised clinical outcomes of cancer management, and adversely affects patients' quality of life (QOL) [82]. The number of cases of neutropenia-associated hospitalization is about 60,000 per year in USA, which is associated with a high death rate of around 6.8% [83].

Neutrophils are one of the first cellular components for inflammatory response and are essential part of innate immune system. Because of significant reduction of neutrophil number, the normal immune response is impaired and the classic sign and symptom for infection is no longer present in cancer patients. Usually, cancer patients only present fever as the only indication of infection during neutropenia. Neutropenia with presence of fever, or febrile neutropenia (FN), is a serious complication caused by chemotherapy, often leading to reduction of dose and treatment delay and hence compromise efficacy of chemotherapy. Furthermore, mortality of cancer patients with FN is significantly increased, especially when patients possess concomitant infection and multiple comorbid diseases [84]. The incidence of FN occurred in Asian early stage breast cancer patients treated with AC (doxorubicin and cyclophosphamide) chemotherapy regimen is around 13.8%, and majority of these patients develop FN when they receive first cycle of treatment [85].

A standard treatment for cancer patients with FN is to give patients broad-spectrum antibiotics [80]. These antibiotics may have toxicity for gastrointestinal, renal, and dermatologic systems. The recombinant human granulocyte colony-stimulating factor (G-CSF) is administered to cancer patients for stimulating proliferation, differentiation and activation of neutrophils. It is important to use G-CSF prophylaxis judiciously to prevent occurrence of FN. Current clinical guidelines recommend administration of G-CSF in chemotherapy regimen with inherent risk of FN that is higher than 20% [86]. The side effects caused by G-CSF include mild-to-moderate skeletal pain which occurs in 20%-25% of patients and allergic reaction [79].

Platelets, also known as thrombocytes, are also sensitive to myelosuppression effect of chemotherapeutic agents. The degree of toxicity on platelets exerted by anti-cancer drugs is lesser than that on WBCs due to the relatively longer life span of platelets that is around 7-10 days. However, patients may develop chemotherapy induced thrombocytopenia if they receive high dose of anti-cancer drugs, multiple cycles of a certain drug or combinational administration of various drugs. National Cancer Institute (NCI) has established hematologic toxicity criteria for thrombocytopenia assessment (Table 1-1). Platelets are responsible for blood coagulation process, thus the reduction in its number (thrombocytopenia) may cause abnormal coagulation and spontaneous bleeding. When platelets count does not recover to normal level during chemotherapy regimen, reduction of dose or delay of treatment may be needed. Thrombocytopenic cancer patients who are bleeding should receive platelets transfusion associated with risk of infection, allergic responses, transfusion-associated lung injury, and alloimmunization.

Red blood cells (RBCs), or erythrocytes, are harmed by myelosuppressive effect of anti-cancer drugs in the least serious degree compared with other blood cell types. The reason is that RBCs have significantly longer circulating time of about 120 days in blood stream than that of WBCs and platelets. However, cancer patients are still at risk for chemotherapy-induced anemia with cumulative cycles of anti-cancer drug administration, which can impact cancer patients' quality of life adversely even at mild to moderate grade. Patients may present fatigue and weakness and feel precluded from a normal life [87]. The main treatment options for anemia induced by anti-cancer drugs include blood transfusions, recombinant human erythropoietin (epoetin alfa), darbepoetin alfa, iron therapy or a combination of various methods. Blood transfusion is warned against by clinicians because of its associated risks including infection, allergic responses, transfusion-associated lung injury, and alloimmunization [88]. Epoetin alfa and darbepoetin alfa may cause side effect including nausea, dyspnea, coughing, weakness, constipation, fever, vomiting and thrombotic events. National Cancer Institute (NCI) has established hematologic toxicity criteria for anemia assessment (Table 1-1). It is critical for doctors to choose most appropriate treatment for chemotherapy-induced anemia based on laboratory test values and patients' clinical status.

Close monitoring of myelosuppression induced by chemotherapy drugs in combination with prevention and treatment of complications, such as neutropenia, thrombocytopenia, anemia, plays an important role in management of cancer patients with chemotherapeutic treatment.

Table 1-1 Common Terminology Criteria for Adverse Events (CTCAE) Adapted from V3.0 NCI-CTCAE

Adverse Event	Grade ^a				
	1	2	3	4	5
Leukocytes (total WBC)	<LLN ^b -3000/mm ³	<3000-2000/mm ³	<2000-1000/mm ³	<1000/mm ³	Death
Neutrophils (ANC ^c)	<LLN-1500/mm ³	<1500-1000/mm ³	<1000-500/mm ³	<500/mm ³	Death
Platelets	<LLN-75,000/mm ³	<75,000-50,000/mm ³	<50,000-25,000/mm ³	<25,000/mm ³	Death
Hemoglobin	<LLN – 10.0 g/dL <LLN – 6.2 mmol/L	<10.0 – 8.0 g/dL <6.2 – 4.9 mmol/L	<8.0 – 6.5 g/dL <4.9 – 4.0 mmol/L	<6.5 g/dL <4.0 mmol/L	Death

^a Grade refers to the severity of the adverse event (AE). Grade 1 Mild; Grade 2 Moderate; Grade 3 Severe; Grade 4 Life-threatening or disabling; Grade 5 Death.

^b LLN: lower limit of normal

^c ANC: absolute neutrophil count

(3) Clinical Measurement and Prediction of Chemotherapy-induced Neutropenia

The complete blood count (CBC) is a commonly used clinical laboratory test to measure hematologic toxicity induced by anti-cancer drugs. The CBC measures white blood cell count, red blood cell count, hemoglobin (Hgb), hematocrit (Hct), mean cell volume (MCV), red cell distribution (RDW), and platelet count. Different types of WBCs (WBC differential) that include neutrophils, lymphocytes, monocytes, eosinophils and basophils, can also be analyzed by CBC. In clinical practice, CBC test is conducted in cancer patients before each cycle of drug administration. Based on the result of CBC and the criteria of hematotoxicity, clinicians adjust treatment dose of next cycle for cancer patients.

The incidence of chemotherapy-induced neutropenia (CIN) is usually predicted based on the potential myelosuppressive effect of chemotherapy regimen. This prediction factor, however, may not be the most effective way to identify cancer patients who are at greatest risk of CIN and hence should be offered with

prophylactic G-CSF administration. In order to apply supportive care measures, such as G-CSF treatment, more cost-effective and targeted, much effort has focused on development of clinical prediction factors and models that can distinguish individuals with high risk of CIN from other cancer patients [89].

So far, various risk factors with predictive value for CIN and its complications have been identified, which can be further classified into three groups: disease-specific, patient-specific and treatment-specific factors.

Disease-specific factors refer to different cancer conditions that can lead to diverse risk of CIN developed in cancer patients. Researchers have found patients with hematologic malignancy develop neutropenia and its complication more frequently than patients with solid tumor [90]. Besides, if the disease is in advanced stage or uncontrolled condition, the patient is more likely to suffer from serious neutropenic complications, including FN or even death [91].

Patient-specific risk factors include age, performance status, comorbidities, and abnormal laboratory test results. Many studies have shown that higher age is a significant independent risk factor for CIN and neutropenia-associated complications. Therefore, senior cancer patients are usually treated with reduced dosage of anti-cancer drugs. Due to the fact that cancer patients with higher age can benefit from full dose of chemotherapeutic agents similarly as young patients [92], it is crucial that effective supportive care is delivered to this population to guarantee treatment efficacy. In addition to advanced age, the poor performance status is also an important risk factor for CIN. Several studies have shown that the presence of other diseases with cancer increases the risk for CIN and neutropenia-related complications. For breast cancer patients, the risk of severe neutropenia and FN is

increased if patients present comorbidities of liver, kidney and heart diseases [93]. Similarly, comorbidities that can lead to increased risk for neutropenia-associated severe complications include hypertension, chronic obstructive pulmonary disease, pneumonia, prior fungal infection, and sepsis [91]. CBC value may serve as predictive risk factor for CIN and its complications. Pretreatment WBCs has been reported to have predictive value for FN in early stage breast cancer patients [93]. If a cancer patient has hemoglobin value of less than 12 g/dL before treatment, this patient is more probably to develop FN in the first cycle of drug administration [94]. Moreover, the response of cancer patients to anti-cancer drug in cycle 1 of chemotherapy regimen has been used to predict CIN in subsequent chemotherapy process by conditional risk models. It has been shown that leukocyte count nadir after first cycle of chemotherapy and decreased level of hemoglobin and platelet count may predict neutropenic complications in subsequent cycles [81, 95].

Since different anti-cancer drugs have variable degrees of myelosuppressive effect, the intensity of one certain chemotherapy regimen can serve as primary determinant of risk of CIN and its complications. For example, high dosage use of anthracycline in early stage breast cancer patients has been identified as an important risk factor for serious neutropenia and FN [96].

Overall, risk factors that have been identified so far for CIN include Patient-specific, Treatment-specific and Disease-specific factors (Figure 1-5).

Figure 1-5 Risk factors for chemotherapy-induced neutropenia. All the risk factors can be divided into three groups: Patient-specific, Treatment-specific and Disease-specific factors.

Several risk models have been developed through integrating various risk factors together to predict CIN and its complications. These risk predictive models, however, have various limitations. Majority of these models were established through retrospective study that were not designed to examine a well-defined hypothesis. These clinical studies were conducted with relatively small sample size and experimental values were not recorded completely. Above all, most of the models are not validated through independent clinical trials and hence most of them are not reliable in their predictive power. Consequently, the clinical application of these risk models is very limited [97].

(4) *Pharmacogenetic Study for Predicting Toxicity before Commencing Chemotherapy*

There are many potential risk factors that can affect toxicity of chemotherapy drug on a certain patient, including combinational use of different drugs, patients' age and gender, renal and liver function, comorbidity and living habits such as smoking and drinking alcohol. However, the inherited genetic factors that can affect pharmacokinetics and pharmacodynamics of drugs play a more important role in determining individual risks. Variation in drug response including drug efficacy and toxicity are resulted from variation in genes encoding proteins responsible for drug metabolism, drug transportation and drug disposition [98]. Pharmacogenetics studies differences in drug response among individuals based on inherited genetic polymorphism [99]. After the term "pharmacogenetics" was first coined in 1950s, a considerable amount of progresses has been made in this field. In the field of cancer chemotherapy, from investigation of single drug response-related candidate gene to genome-wide linkage analyses, pharmacogenetic research aims to predict drug response of tumor and normal tissue for chemotherapeutic agents based on genetic polymorphism and hence facilitate personalized chemotherapy regimen which is giving most appropriate drug at optimal dosage to each cancer patient to avoid adverse reaction and achieve maximal efficacy [100].

Drug response-related genes refer to genes that encode drug transporters, drug metabolizing enzymes, targeting proteins of anticancer drugs, apoptosis relevant proteins and chemokines. These candidate genes can be classified into three groups based on how they influence the effect of chemotherapeutic agents (Figure 1-6) [100]. Correlation analysis between genetic polymorphism of these candidate genes and pharmacokinetics and pharmacodynamics of chemotherapy drugs can help to identify biomarkers that have predictive value for adverse drug reaction. Successful examples with clinical significance include identification of thiopurine

methyltransferase (TPMT) genotype effect on hematopoietic toxicity of mercaptopurine (6-MP), dihydropyrimidine dehydrogenase (DPD) genotype effect on 5-fluorouracil (5-FU) toxicity and UDP-glucuronosyltransferase 1A1 (UGT1A1) genotype effect on irinotecan toxicity [101]. Doxorubicin and taxanes are the first-line anti-cancer drugs for breast cancer treatment. It was reported that differences in pharmacokinetics and pharmacodynamics of doxorubicin might be resulted from polymorphic genotype of human carbonyl reductase CBR3 among individuals and ethnic groups [102]. Genetic variability in genes encoding proteins participating in taxanes transport and clearance reduces drugs excretion, and thus drug toxicity is increased [103].

Figure 1-6 Drug response-related candidate genes [100]. Reprinted from ref [100] with permission from Elsevier.

Although some promising results have been obtained through single gene pharmacogenetic study, drug response of most anti-cancer drugs is probably

multigenic incidence that depends on interplay among multiple polymorphic genes. Therefore, the candidate gene approach that study single genes is not suitable for majority of chemotherapy drugs.

Following remarkable progress in genome-wide genotyping and sequencing technology, pharmacogenetics has shifted from single gene evaluation to genome-wide genetic polymorphism study. As the modern form of pharmacogenetics, pharmacogenomics determines the heritability of drug toxicity by high-throughput molecular technologies on genome-wide scale. Common single-nucleotide polymorphisms (SNPs) throughout the whole genome can be rapidly evaluated for the correlation with pharmacological traits. The process of cancer pharmacogenomic studies is shown in Figure 1-7 [104].

Some successful genome-wide association studies (GWAS) with clinical relevance for chemotherapy toxicity have been reported. However, genome-wide pharmacogenomics is still facing lots of challenges. The complexity includes identification of relevant patient populations, understanding of the extent of linkage disequilibrium at a particular gene locus and contribution of environmental factors in certain trait expression. Additionally, a huge sample size is required to identify a gene of interest from entire genome scanning, which is far beyond the patient number in common clinical trials [101].

Figure 1-7 Process of cancer pharmacogenomics research for identifying polymorphic genes with predictive value for anti-cancer drug toxicity [105]. Reprinted from ref [105] with permission from John Wiley and Sons.

Treatment decision of chemotherapy regimen and drug dosage base on genotype information of cancer patients is still limited in clinical practice due to lack of established pharmacogenetic recommendation. There are various problems that need to be solved in the field of pharmacogenetics. The classification of heterogeneous phenotypes is not accurate enough for establishment of correlation between genetic biomarkers and complex traits. It will take a long time to evaluate the clinical value of pharmacogenetic biomarkers in retrospective and prospective clinical trials and many candidate genes may drop out. A considerable number of genetic polymorphisms can be identified through genome-wide genotyping. It is complicated to recognize candidate gene with prediction value and find out combined effect of multiple low-risk polymorphisms. In order to predict drug toxicity more reliably, it is necessary to consider gene expression and enzyme activity together with genotype, which increases the complex of pharmacogenetic research. Genetic polymorphisms are distributed differently with variable frequencies among diverse ethnic groups. The result obtained from one ethnic group might not be applicable for other ethnic populations [100].

Given that various challenges pharmacogenetic study have not been overcome, the implementation of pharmacogenetics to clinical practice is still very limited. To be realistic, we are still at the initial stage of genetics-based chemotherapeutic strategy that can avoid side effect of chemotherapy drugs in maximal extent.

(5) Biomarkers in Blood with Predictive Value for Hematotoxicity of Chemotherapy Drug

A few studies have been conducted with the aim of searching for soluble proteins in the blood that can serve as predictor of hematotoxicity induced by anti-cancer drugs. Through one clinical study, in which patients with lymphoma were treated with doxorubicin and cyclophosphamide, the level of p75 tumor necrosis factor and its soluble receptor in baseline plasma sample was identified as predictor for myelosuppression induced by chemotherapy [106]. However, this predictive factor lacked validation from other studies. In another study, proteomics technology was applied to examine baseline plasma proteome of advanced pancreatic cancer patients receiving gemcitabine. The amount of haptoglobin exhibited the most significant difference between group with hematologic adverse events and group without. Therefore, the decreased level of haptoglobin may have the predictive value of gemcitabine-induced hematologic toxicities [107]. However, the underlying mechanism of why the level of haptoglobin can correlate with occurrence of hematologic adverse event needs further clarification. Besides the predictive value of haptoglobin needs to be confirmed by other independent clinical trials.

Overall, more biomarkers with high specificity and sensitivity are needed to be identified for accurate measuring and prediction of hematotoxicity induced by chemotherapeutic agents in cancer patients.

1.3 Cell Metabolic Pathways and Enzyme Related to Cancer Metabolism

Normal cells favor mitochondrial oxidative phosphorylation pathway to produce energy, which is more efficient than aerobic glycolysis in terms of adenosine triphosphate (ATP) generation. However, most cancer cells rely on aerobic glycolysis even in the presence of sufficient oxygen to generate necessary energy. This observation was termed Warburg effect that has been well known and widely accepted for several decades [108]. The pentose phosphate pathway (PPP) that branches from glycolysis has attracted more and more attentions in recent years as this metabolic pathway does not only satisfy cancer cells' anabolic requirements but also plays an essential role to generate intracellular antioxidants that enable glycolytic cancer cells to combat oxidative stress (Figure 1-5) [109].

1.3.1. Pentose Phosphate Pathway (PPP) in Cells

The pentose phosphate pathway (PPP) is an important metabolic pathway in cells. It is primarily a catabolic pathway that takes place in cytoplasm of both prokaryotes and eukaryotes, during which NADPH, an important intracellular antioxidant, is generated to act as reducing power in cells and participate in various biosynthesis reactions, such as fatty acid synthesis, steroid hormone biosynthesis and cholesterol biosynthesis [110]. The PPP also can work as an anabolic pathway to generate ribose-5-phosphate (R5P) that is precursor for nucleotide biosynthesis. This metabolic pathway begins with glucose and contains multiple steps, which can be divided into two distinct phases: oxidative phase and non-oxidative phase. The oxidative phase of PPP contains 4 irreversible reactions, in which two molecules of

NADPH are generated during the oxidation of glucose into ribose-5 phosphate. These reactions are primarily catalyzed by two enzymes: glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (PGD) [111]. Following oxidative reactions, non-oxidative phase contains a series of reversible reactions, in which the carbon skeletons of various carbohydrates, such as ribose-5-phosphate, glyceraldehyde-3-phosphate and fructose-6-phosphate, are rearranged based on the needs of cell. Overall, as the only source of NADPH in cytoplasm, PPP is a major antioxidant defense system of cells.

Figure 1-8 The oxidative and non-oxidative phase of pentose phosphate pathway (PPP). Reprinted from ref [109] with permission from Springer Berlin Heidelberg.

1.3.2. Glucose-6-Phosphate Dehydrogenase (G6PD)

(1) General functions of G6PD

Human glucose-6-phosphate dehydrogenase (G6PD) is a cytosolic enzyme that catalyzes the first step of oxidative branch of PPP. Encoded by a housekeeping X-linked gene, G6PD is famous for its genetic mutations that result in an inherited disorder called G6PD deficiency [112]. As the rate-limiting enzyme of PPP, the main function of G6PD is to provide reducing power (NADPH) for cellular antioxidant defense and pentose phosphates for fatty acid and nucleic acid synthesis through promoting the whole efficiency of PPP [113].

(2) G6PD regulation

There are multiple regulators that either positively or negatively regulate G6PD. Nuclear-factor-E2-related factor (Nrf2), which is a transcription factor that regulates expression of various antioxidant proteins, initiates the transcription of G6PD under oxidative stress [114]. TP53-induced glycolysis and apoptosis regulator (TIGAR) was reported to activate G6PD and PPP and result in a decrease of intracellular ROS level [115]. When cell requires increased level of NADPH for lipid biosynthesis, G6PD is positively regulated by sterol responsive element binding protein (SREBP) that is a major lipid transcriptional activators [116]. The other positive G6PD regulators include S6 kinase, mTOR (mammalian target of rapamycin), insulin, vitamin D, AKT and PI3-K [117].

The tumor suppressor p53 was shown to negatively regulate G6PD activity. It was reported that, in colon cancer cells, p53 was able to bind with G6PD directly in cytoplasm and prevent the formation of G6PD dimer/tetramer and thus caused decrease of G6PD activity [118]. G6PD activity is also inhibited by tumor necrosis factor alpha [114]. The cyclic AMP (cAMP) may reduce G6PD activity by both interfering with G6PD transcription and modifying protein structure of G6PD [119].

(3) *G6PD and redox homeostasis*

Cell survival and normal cellular functions are largely dependent on a proper redox homeostasis in cells. An increased level of oxidative stress is usually observed in cancer cells [120]. When the generation of reactive oxygen species (ROS) exceeds its elimination, ROS is accumulated in cells and results in intracellular oxidative stress. There are various types of ROS that include the superoxide ($\text{O}_2^{\bullet-}$), hydroxyl free radicals ($\text{HO}^\bullet$) and hydrogen peroxide (H_2O_2) that is a non-radical molecule. Due to oxygen contained in these chemicals and their reactive properties, they are broadly called ROS.

The oxidative stress can be induced in cells by different conditions. Hypoxia, which is caused by insufficient oxygen supply, is one of ROS inducers. Hypoxic stress is caused by absence of nutrient-supplying blood vessels, increased distance between blood flow and tissue or reduced oxygen supply due to anemia. Increased ROS production in mitochondria is stimulated by hypoxia [121]. Cancer cells require high ATP level to support their sustained growth and proliferation. Therefore, they maintain a high level of metabolism. ROS, as the by-products of mitochondrial respiration, is accumulated excessively and thus leads to oxidative stress [122]. The post-translational modification and formation of correct conformation of nascent proteins take place in endoplasmic reticulum (ER). When proteins are misfolded in ER, the unfolded protein response (UPR) and ER stress is elicited and finally lead to ROS accumulation [123].

Cancer cells are able to develop an increased level of ROS scavengers to counteract the excessively increased ROS level [124]. As the most plentiful intracellular antioxidant molecule, glutathione (GSH) is fundamental for redox

homeostasis within the cell and hence cell survival [125]. ROS can be directly eliminated by GSH with the catalysis by glutathione *S*-transferase (GST) and glutathione peroxidase (GPX). Many tumor types have been found to modify their GSH metabolism.

Besides GSH synthesis through γ -glutamyl cycle catalyzed by GSH synthetase (GSS), the other important way to produce GSH by cells is to recycle reduced GSH. In the presence of reducing agent NADPH, the oxidized glutathione disulfide (GSSG) is reduced by glutathione reductase (GSR) into GSH [126].

As the G6PD-controlled PPP provides cells with NADPH, it is expected that the elevated G6PD activity can protect cells from oxidative stress-induced cell damage by generation of reducing power that is NADPH and GSH.

Figure 1-9 G6PD facilitates cell to maintain redox homeostasis [126]. Reprinted from ref [126] with permission from Bentham Science Publishers Ltd.

(4) *The role of G6PD in cancer*

Regeneration of GSH by G6PD enables cells to detoxify ROS and hence promotes cancer cell survival [115]. G6PD is found to up regulate by hypoxia that usually occurs in the central part of solid tumor due to rapid cell growth. GSH and N-acetylcysteine can abrogate this up-regulation [127]. Inadequate G6PD activity makes cells more sensitive to apoptosis induced by oxidative stress [128]. Similarly, inhibition of G6PD activity promoted cell death mediated by H₂O₂. In contrast, overexpression of G6PD enables cells to become resistant to apoptosis [129]. As a tumor suppressor, p53 is reported to directly inactivate G6PD through hindering the formation of G6PD active dimers [118]. When p53 is mutated in tumors, the deregulated activation of G6PD shuttles more glucose into PPP and thus favors cancer cell survival. The activity of the G6PD has been observed to be 40% higher in the daunorubicin-resistant P388 murine lymphocytic leukemia cells than in daunorubicin-sensitive P388 cells (12). Similarly, doxorubicin-resistant human colon cancer cells (HT29-DX) exhibit decreased drug accumulation due to the higher activity of G6PD (13), showing that elevated levels of NADPH and GSH maintained by the G6PD play a critical role in anti-cancer drug resistance.

The expression level of G6PD has been reported to increase in various cancer lesions, especially in cancers with advanced stages and metastatic disease, which is associated with poor treatment outcome [130-133].

1.4 Project Rationale

Chemotherapy is the backbone of systemic treatment for TNBC, among which anthracyclines are important agents and represent the current standard treatment option [26, 27].

However, treatment failure usually happens due to the drug resistance developed by TNBC cells. Especially when metastasis occurs, the first line anthracycline drug for TNBC treatment, DOX, usually loses its efficacy. Therefore, the metastatic breast cancer (MBC) remains almost an incurable disease with a five-year survival probability of only around 25%. More than 90% of MBC patients suffer from treatment failure due to chemotherapy resistance. The mechanism underlying the metastasis acquired DOX resistance of TNBC cells remains to be elucidated. The first part of study focuses on exploring drug resistance mechanism developed by TNBC cells towards DOX treatment. Through illuminating the possible molecular players involved in DOX resistance, the drug efficacy of DOX would be improved by interfering with the defense mechanism developed by metastatic TNBC cells and thus the survival probability of TNBC patients with metastatic disease would be increased.

Another challenge the clinicians are facing during anthracycline administration for breast cancer treatment is the dose limiting drug toxicity, which often leads to treatment delay or even discontinuation and hence the treatment failure. Manifested by dose-dependent neutropenia, hematologic toxicity is the most common acute DOX-associated dose-limiting toxicity, which is closely associated with failure of treatment and an inevitable consequence of non-selective effects of anthracyclines on both cancerous and healthy cells. Hematologic toxicity is dose-dependent, but sensitivity to myelotoxicity varies among individuals and there is currently no

reliable predictor of hematologic toxicity. The complete blood count (CBC) is a commonly used clinical laboratory test to measure hematologic toxicity induced by anti-cancer drugs. However, by the time a fall in white blood cell (WBC) count is detected, little can be done other than administering granulocyte-colony stimulating factor (G-CSF) to boost WBC production and empirical antibiotics if fever is also present. This is less than satisfactory. The objective of the second part of the research is to develop a direct and simple method to assess hematologic toxicity in cancer patients and to potentially predict hematologic adverse event at early stage of treatment regimen. It is expected that this indicator to be developed is able to predict hematologic adverse event at early stage of chemotherapy course and hence can guide dose schedules of anthracyclines and also supportive care given to cancer patients receiving anthracyclines. Therefore, the safety of anthracycline administration would be improved.

Overall, through reducing drug resistance developed by TNBC toward anthracyclines to improve drug efficacy and also monitoring the hematologic toxicity associated with anthracyclines to promote the safety of drug use, the benefit of anthracycline administration for TNBC patients is expected to be maximized. The treatment failure of anthracycline administration in TNBC patients, especially in metastatic disease, could be reduced in the future clinical practice.

1.5 Scope of Thesis

Chapter 1, this chapter, introduces all background information of the whole research project and states the motivation and objective of this thesis. The remaining chapters are organized as follows:

Chapter 2 describes all methods and materials that were applied in this research project.

Chapter 3 introduces the novel TNBC cell lines used in this project, describes the drug treatment results of these cells and focuses on the 2D-DIGE proteomics result and its analysis.

Chapter 4 describes validation on the function of G6PD in DOX resistance and explains how high expression of G6PD induces DOX resistance in metastatic as well as original TNBC cells. The content of Chapter 3 and chapter 4 is a version of a to-be-submitted manuscript.

Chapter 5 presents the development of plasma caspase-3 level in cancer patients as the indicator as well as potential predictor for hematologic toxicity of anti-cancer drugs.

Chapter 6 concludes this thesis and discusses possible directions for further work.

Chapter 2 Materials and Methods

2.1 Cell Culture

MDA-MB-231 cells were provided by Professor Xiaofeng Le from Department of Experimental Therapeutics at The M.D. Anderson Cancer Center, University of Texas. MDA-MB-231-C3 (231-C3) cells were generated by transfecting plasmid encoding the fluorescence resonance energy transfer (FRET)-based caspase-3 biosensor into MDA-MB-231 cells [134, 135]. MCF10A, MDA-MB-157, BT-549 and MDA-MB-468 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MCF10A cells were cultured in MEGM mammary epithelial cell growth medium (Lonza, Alpharetta, GA, USA), while other cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Buckinghamshire, UK) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). All cells were cultured in 10 cm petri dishes (TPP, USA) and maintained in a humidified incubator with 5% CO₂ at 37°C. Cells were passaged every 2 to 3 days when it reached around 80% confluence with passage ratio of 1:5 to 1:3 for most of cell lines.

2.2 Detection of Cell Apoptosis by FRET Imaging and Cell Viability by MTT Assay

The FRET imaging and MTT assay were applied to evaluate the apoptosis-inducing and proliferation-inhibiting effects of DOX on cancer cells. Briefly, 5000 cells in

100µL of culture media were seeded into each well of a 96-well plate with triplicate wells for each drug concentration group. After appropriate treatment, cells were imaged with a motorized fluorescence microscope (Axiovert S 100, Carl Zeiss, Germany) equipped with FRET filters ($Ex=436 \pm 10$ nm; dichroic mirror = 455 nm; $Em_1 = 480 \pm 20$ nm and $Em_2 = 535 \pm 15$ nm), a computer-controlled camera (AxioCam MRm, Carl Zeiss, Germany) and operated by Zen 2012 software (Carl Zeiss). At least 5 pictures were captured for each group. The representative pictures were selected out and shown. The apoptotic rate was quantified from FRET images.

After FRET imaging, the cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. MTT solution was prepared by dissolving MTT power (Sigma-Aldrich) in phosphate buffered saline (PBS) at concentration of 5 mg/mL. 10 µl of MTT solution was added into each well and then the plate was incubated at 37°C for 3 hours to allow the reduction of MTT into purple-coloured formazan. After incubation, 100 µl of 10% sodium dodecyl sulphate (SDS) solution containing 0.01 M hydrochloric acid (HCl) was added into each well to facilitate the dissolution of the formazan purple crystals. The optical density of solution in wells of 96 well plate at 595 nm was determined using a plate reader (Perkin-Elmer, USA) after the 96 well plate is incubated at 37°C overnight.

2.3 Detection of Intracellular ROS Level

MDA-MB-231 cells with or without 10 µM DOX treatment for 24 h were stained with 10 µM CM-H₂DCFDA (Thermo Fisher Scientific, Waltham, MA, USA) in FluoroBrite medium (Thermo Fisher Scientific, Waltham, MA, USA). After incubation for 20 min, the cells were immediately imaged using an inverted

fluorescence microscope (Axiovert S 100, Carl Zeiss, Germany). At least 5 pictures were captured for each group. The representative pictures were selected and shown. To quantify the ROS generation, MDA-MB-231 cells were treated with or without DOX in a 60 mm dish. After stained with 10 μ M CM-H₂DCFDA and washed with PBS for twice, cells were collected and resuspended in FluoroBrite medium. The intensity of DCF fluorescence was immediately determined by a LSR Fortessa X-20 flow cytometer (BD, USA). In addition, cells without drug treatment were stained with 10 μ M CM-H₂DCFDA and subjected to flow cytometry analysis to serve as negative control of this study.

2.4 2-D DIGE Proteomics Analysis

One dish (10 cm) of 231-C3 or 231-M1 cells in 70% confluence were collected by cell scratcher in cold PBS on ice respectively. Cells were centrifuged to remove all liquid and the cell pellets were kept at -80°C before sending to Applied Biomics (Hayward, CA) in dry ice for 2-D DIGE and mass spectrometry protein identification by MALDI-TOF and tandem MS. Briefly, cells were lysed with 2-D lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.8) for protein extraction. After measuring and adjusting protein concentration, equal amount of protein samples was labeled with fluorescent size and charge matched CyDye. Protein sample from 231-C3 cells was labeled with Cy2 and that from 231-M1 cells was labeled with Cy3. After labeling, two samples were mixed and about 80 μ g of sample was loaded onto one electrophoresis gel (analytical gel) and separated by 2D-DIGE. The 2D gel was scanned using a Typhoon Trio scanner (Amersham BioSciences, Piscataway, NJ). Images were then analyzed by Image Quant software (Amersham BioSciences). All differentially expressed proteins were

further analyzed from the 2D-DIGE-derived data using Decyder software (Amersham BioSciences). Totally 54 protein spots of interest were selected from the analytical gel. For protein identification, about 600 µg of unlabeled protein and a small amount of labeled proteins were loaded and separated in a preparation 2D gel (prep gel). The spots of interest were picked up by Ettan Spot Picker (Amersham BioSciences) based on the in-gel analysis and spot picking design by DeCyder software. The gel spots were washed a few times then digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The digested tryptic peptides were desalted by Zip-tip C18 (Millipore). Peptides were eluted from the Zip-tip with 0.5 ul of matrix solution (α -cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on the AB SCIEX MALDI plate (Opti-TOFTM 384 Well Insert).

MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Both of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the database of National Center for Biotechnology Information non-redundant (NCBI nr) or Swiss-Prot database. Searches were performed without constraining protein molecular weight or isoelectric point, with

variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Candidates with either protein score C.I.% or Ion C.I.% greater than 95 were considered significant.

Gene IDs of these identified 54 proteins were further analyzed by Panther Classification System against NCBI (*Homo sapiens*) dataset. Proteins were classified in terms of molecular function, protein class and biological process.

2.5 Western Blot Analysis

The cells with or without treatments were collected and lysed with RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, MO, USA). The protein concentration was determined using a Bradford assay kit (Bio-Rad, Berkeley, CA, USA). Protein in cell lysate was separated by SDS-PAGE gels and was transferred to a PVDF membrane. After blocked with low-fat milk, the membrane was incubated with primary antibody (1:1000 dilution) overnight at 4°C. The protein blots were further probed with HRP-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature and developed in ECL solution (Thermo Scientific, USA). The intensities of the bands on the membrane were measured using Image Pro Plus (Media Cybernetics, USA).

2.6 Clinical Data Analysis

The expression data of G6PD in clinical samples was taken from the study by Curtis et al (2012) [136]. The dataset was generated by transcriptional profiling on

the Illumina HT-12 v3 platform, followed by normalization. It is stored on European Genome-Phenome Archive (EGA, <http://www.ebi.ac.uk/ega/>), under accession number EGAS00000000083. The clinical annotations for normal samples and TNBC samples are provided in the supplementary information from the same study. The boxplot was generated in R using ggplot2 package. Welch's t-test for comparing the mean of G6PD expression between normal and TNBC samples was performed by ggpubr package.

Clinical follow-ups and G6PD expression data were obtained from the same above-mentioned study. The R packages survival and survminer were used to generate the Kaplan-Meier plot. Patients were divided to G6PD high and low expression groups by applying a cutoff of 60 percent quartile on normalized expression values. Mantel-Haenszel logrank tests were used to compare the overall survival probabilities of two subgroups of TNBC patients and to compute R value.

2.7 Knockdown and Inhibition of G6PD

Validated Silencer Select siRNA against G6PD (siRNA ID s5447) and Silencer Select Negative Control siRNA were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cells were seeded into 60-mm dishes for overnight culture before transfection. After washed twice with PBS, cells were transfected with 20 nM siRNA in OptiMEM (Life Technologies) using Lipofectamine RNAiMAX (Thermo Fisher Scientific), according to the manufacturer's instructions. After incubation for 6 h, the transfection complex was replaced with regular complete DMEM. After 45 h, the cells were subject to further experiments. To inhibit the activity of G6PD, 200 μ M of DHEA (Sigma-Aldrich) was used to pre-treat the cells

for 12 h before they were subjected to further treatment. DHEA continuously existed for the whole duration of drug treatment.

2.8 Detection of Intracellular DOX Concentration

Cells were seeded in 60-mm dishes and then incubated in phenol-red free DMEM containing 5 μ M DOX for different time periods. After DOX treatment, cells were washed twice in ice-cold PBS and harvested. Cell pellets were obtained by centrifuge at 800g for 3 min at 4°C. Cells were then lysed in RIPA buffer and subjected to sonication. A small portion of cell lysate was measured for cellular protein concentration using a Bradford assay kit (Bio-Rad, Berkeley, CA, USA). The remaining cell lysate was mixed with acidified isopropanol (0.75 M HCl) (v/v 1:3) to extract DOX. After thorough vortex and overnight incubation at -20°C, the mixture was centrifuged at 15,000g for 10 min. Fluorescence of organic phase was determined by a spectrofluorimeter (SpectraMax M5, Molecular Devices). Excitation and emission wavelengths were at 480 and 590 nm respectively. Fluorescence intensity was converted to amount of DOX by plotting a standard curve. The amount of DOX (nmol) was normalized to amount of cellular protein (milligram).

2.9 GSH Level Determination

Cells with or without G6PD silencing were grown in 60-mm dishes. After 5 μ M DOX treatment for different time periods, cells were collected and lysed in PBS / 0.5% NP-40. The reduced GSH level was determined using the GSH/GSSG ratio detection assay kit (ab138881, Abcam, USA). The excitation and emission

wavelengths of fluorescence were at 490 nm and 520 nm respectively, which was determined by using a spectrofluorimeter (SpectraMax M5, Molecular Devices).

2.10 Caspase-3 Assay

The caspase-3 activity of plasma was determined by the methods developed by Prof. Kathy Qian Luo's group [137, 138]. Briefly, in each well of a 96-well plate, 25 μ l of plasma was mixed with 225 μ l of reaction buffer and 0.5 μ l of caspase-3 substrate, Ac-DEVD-AMC (1 μ g/ μ l). The activated caspase-3 reacted with its substrate by cleaving the bond linking peptide DEVD and AMC group. Thus, AMC group was released and emitted fluorescence at 460 nm when excited by light at 360 nm. The reaction mixture was incubated at 37°C in dark for 24 hours, and the fluorescence intensity was measured in a spectrofluorometer using the Ex = 360 nm and Em = 460 nm.

The caspase-3 activity was measured in triplicate and repeated for three times. The average level of caspase-3 activity was normalized to baseline sample and compared within the same patient during the whole course of chemotherapy and among patients receiving various treatment regimens.

2.11 Preclinical Study

A pilot animal study was conducted using mouse (BALB/c, female, 6-8 weeks). Paclitaxel was administered by a single i.v. bolus injection into the tail vein of mouse at 25 mg/kg. The drug was given every 4 days for 6 cycles, and blood collection was scheduled after 72 h of each cycle of drug administration through cardiac puncture. Plasma sample was examined by caspase-3 assay.

2.12 Clinical Study

A pilot study was conducted involving 40 women with non-metastatic breast cancer; 20 who received chemotherapy in the neoadjuvant setting (prior to surgery) and 20 in the adjuvant setting.

Different chemotherapy regimens that were given to breast cancer patients are shown as following:

The most commonly used AC-T regimen: doxorubicin 60 mg/m² in combination with cyclophosphamide 600 mg/m², given every 21 days for 4 cycles, followed by paclitaxel (Pac) 75 mg/m², given every 7 days for 12 cycles.

TC regimen: docetaxel 75 mg/m² in combination with cyclophosphamide 600 mg/m², given every 21 days for 4 cycles.

Schedule of blood taking was coincided with their routine blood taking:

AC-T regimen: S1 - baseline, prior to the start of AC, S2 - prior to 3rd cycle of AC, S3 prior to the start of Pac, S4 - after 6 cycles of Pac, S5 - at the completion of Pac.

TC regimen: S1 - baseline, prior to the start of TC, S2 - prior to 3rd cycle of TC, S3 - at the completion of TC.

Current indicators of toxicity were also recorded, including trends in white blood cell (WBC) count including absolute neutrophil count (ANC), hemoglobin levels and platelet count. In addition, neutropenia episodes requiring G-CSF support, febrile neutropenia requiring hospitalization and the degree of peripheral neuropathy (both sensory and motor) was recorded. In instances where the

chemotherapy was given in a neoadjuvant setting, changes in the tumor volume or extent (either clinically or radiological) were also recorded.

The whole blood samples obtained from cancer patients were subjected to processing shown as follows:

1. Spin the sample at 1200 g for 15 minutes at 4°C with brake-off in a horizontal, swinging bucket centrifuge.
2. Label the cryovials while centrifuging. The plasma will be on top of the whitish layer or buffy coat.
3. Carefully transfer the plasma into a 15 ml falcon tube without disturbing the buffy coat layer. If more than one tube is drawn, pool the plasma and mix. Collect plasma fraction to within 0.1 ml of the interface layer. Discard the remaining pellet.
4. Aliquot 100-250 µl of plasma into each labeled cryovials. Record the volume. This process should be completed within 1 hour of centrifugation.
5. Check that all aliquot vial caps are secure and that all vials are labeled.
6. Place all aliquots upright in a specimen box and store at -80°C.

Caspase-3 activity of each clinical sample (plasma from cancer patients) was measured through caspase-3 assay, which was further normalized with the baseline sample. Relative percentages and raw data of all the samples were analyzed and compared with WBC counts. The incidence of significant increase of caspase-3 activity was calculated and compared among different treatment groups. The correlation study was conducted between increase of caspase-3 activity and hematologic adverse event, such as neutropenia, febrile neutropenia, induced by chemotherapeutic agents in cancer patients.

2.13 Statistical Analysis

All data are represented as the mean $\pm$ SD of at least three independent experiments. Statistical significance was analyzed using Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Chapter 3 Identification of Proteins

Associated with Doxorubicin Resistance

of Triple Negative Breast Cancer Cells

3.1 Introduction

Treatment for triple negative breast cancer (TNBC) remains a big challenge in clinical practice. Because TNBC cells do not express either estrogen receptor (ER) or progesterone receptor (PR) and cells do not overexpress HER2 (human epidermal growth factor receptor 2), they respond poorly to either hormone therapy or HER2 targeted therapy [139]. Chemotherapy is the backbone of systemic treatment for TNBC, among which anthracyclines are important agents and represent the current standard treatment option [26, 27]. Several anthracycline antibiotics are available now, among which doxorubicin (DOX) is currently considered as one of the most effective drugs to treat breast cancer. However, the early relapse of TNBC that leads to devastating consequences of cancer patients and the premature development of drug resistance that causes unsuccessful treatment outcome were observed by clinicians [32, 33]. Compared with other types of breast cancer, TNBC has higher rate of relapse with most recurrence occurring in 3 years of diagnosis and early visceral metastasis [32, 34]. The breast cancer cells can spread to many sites of body, including lung, bone, liver, pleura, adrenal glands, etc., among which around 65% of distant metastasis involves lung [140]. Unfortunately, the metastatic breast cancer (MBC) remains almost an incurable disease with a five-

year survival probability of only around 25%. More than 90% of MBC patients are suffered from treatment failure due to chemotherapy resistance [27].

Although DOX has been used for treating a wide range of cancers for more than 4 decades, the exact mechanism of action underlying cancer cell killing effect of DOX remains uncertain and controversial. Among various proposed mechanisms, which include intercalation into DNA, disturbing the function of topoisomerase, redox cycling in mitochondria and reactive oxygen species generation (ROS) generation etc., the ability of inducing intracellular oxidative stress attracts interest from a huge population of scientists [45-48]. More specifically, DOX could be oxidized to semiquinone and when semiquinone is converted back to the original molecule, reactive oxygen species (ROS) are generated that lead to increased intracellular oxidative stress [52]. When the oxidative stress overwhelms the antioxidant ability of cancer cells, cell apoptosis is triggered [53].

One previous study in our research group shows that ROS generation is one of the mechanisms of DOX-induced cancer cell apoptosis and an antioxidant protein expressed in mitochondria, manganese superoxide dismutase (MnSOD) is involved in the DOX resistance [14]. Through literature study, another antioxidant protein expressed in cytoplasm, G6PD was reported to be up-regulated in mice brain metastasis compared with original circulating tumor cells from breast cancer patient [130]. High G6PD expression was also reported to associate with lymph node metastasis and distant metastasis in gastric cancer [131]. However, the role of G6PD involved in metastasis-acquired drug resistance remains to be elucidated.

In this chapter, 2D-DIGE proteomics was applied to compare the protein profiles between metastatic and original TNBC cells to explore possible antioxidant proteins as the major player leading to DOX resistance of metastatic TNBC cells.

3.2 Results

3.2.1. Establishment of Metastatic TNBC Cell Line

An engineered TNBC cell line, 231-C3 that expresses fluorescent apoptosis sensor was previously established. 231-C3 cells appear green when alive and blue when undergo apoptosis due to the reduction of fluorescence resonance energy transfer (FRET) between the donor, cyan fluorescent protein (CFP), and the acceptor, yellow fluorescent protein (YFP) of apoptosis biosensor expressed inside cells. 231-C3 cells were injected into tail vein of an immunodeficient mouse. After 45 days, a micro-metastasis growing on the lung of the mouse was dissected for primary culture and a novel lung metastatic TNBC cell line, 231-M1 was generated [14] (Figure 3-1).

Figure 3-1 Schematic diagram of 231-M1 cells generation. 231-C3 cells were injected into tail vein of a nude mouse. After 45 days, a micro-metastasis was formed in the lung of the mouse and dissected for primary culture and a novel metastatic TNBC cell line, 231-M1 was generated.

3.2.2. Metastatic TNBC Cells Developed Resistance to DOX Treatment

231-C3 and 231-M1 cells displayed significant difference when they were treated with DOX *in vitro*. 231-C3 cells displayed a dose and time dependent response to DOX treatment (Figure 3-2A) and when the concentration is above 5 μM , obvious cell apoptosis was triggered as revealed by FRET imaging (Figure 3-2C). Compared with 231-C3 cells, 231-M1 cells are less sensitive to DOX treatment, exhibiting significantly higher cell viability and less cell apoptosis (Figure 3-2A and 3-2B). In particular, after 24 h of DOX treatment at 5 μM and 10 μM , the viability of 231-M1 cells was 71% and 67% respectively, which is significantly higher than the viability of 231-C3 cells, which were 46% and 38%, respectively. When treatment time extended to 48 h, 231-M1 cells exhibited more than 2-fold higher cell viability compared to that of 231-C3 cells upon DOX treatment at 5 μM and 10 μM (Figure 3-2A). As mentioned above, in FRET imaging, cells undergoing apoptosis show blue color, whereas living cells display green color. Only 11%, 21% and 25% of 231-M1 cells were undergoing apoptosis after 24 h of DOX treatment at 2.5 μM , 5 μM and 10 μM respectively, which is significantly less than over 50% of apoptotic rate in 231-C3 cells (Figure 3-2B and 3-2C).

Figure 3-2 Metastatic 231-M1 cells develop resistance to DOX treatment. (A) 231-M1 cells displayed significantly higher cell viability than 231-C3 cells upon DOX treatment. The cells were treated with DOX at 2.5 μM, 5 μM and 10 μM respectively for 24 h before subjecting to MTT assay. The results were normalized to the control group without drug treatment. (B) 231-M1 cells exhibited significantly lower cell apoptotic rate than 231-C3 cells upon DOX treatment. The cells were treated with DOX at 2.5 μM, 5 μM and 10 μM respectively for 24 h before subjecting to FRET imaging. Apoptotic rate of these sensor cells was quantified from FRET images. (C) Representative FRET images of 231-C3 and 231-M1 cells after 24 h of DOX treatment at 2.5 μM, 5 μM and 10 μM respectively as well as without DOX treatment. Scale bar, 100 μm.

3.2.3. Doxorubicin Induces High Level of Intracellular Oxidative Stress

To figure out why 231-M1 cells developed drug resistance towards DOX treatment compared to 231-C3 cells, the mechanism of action of DOX was investigated. Many studies suggested DOX could induce high level of oxidative stress inside cells [45-48]. In current study, a general oxidative stress indicator,

CM-H₂DCFDA, was used to detect oxidative stress level inside cells during DOX treatment. Oxidation of this indicator inside cells leads to increase of green fluorescence that can be imaged by fluorescence microscope and quantified by flow cytometry. MDA-MB-231 cells treated with DOX for 24 h at 10 μ M emitted strong green fluorescence (Figure 3-3A). Flow cytometry results presented a significant shift from control to DOX treated cells (Figure 3-3B), while quantification revealed a 2.3-fold increase of fluorescence intensity (Figure 3-3C). These results indicate high level of intracellular oxidative stress was generated by DOX.

Figure 3-3 DOX generates high level of intracellular oxidative stress in MDA-MB-231 cells. (A) Representative fluorescent images of MDA-MB-231 cells that were treated with or without 10 μ M of DOX for 24 h and stained with CM-H₂DCFDA. Scale bar, 100 μ m. (B) Flow cytometry analysis of these stained cells and cells without staining (Untreated). (C) Quantified fluorescence intensity from flow cytometry analysis. The results were normalized to control group with DCFDA staining alone.

3.2.4. Identification of Proteins Associated with Doxorubicin Resistance by Two-dimensional Difference Gel Electrophoresis (2D-DIGE) Proteomics

Because 231-M1 cells survived better than 231-C3 cells upon DOX treatment that induces high level of oxidative stress inside cells, I hypothesized that 231-M1 cells had higher antioxidant ability over 231-C3 cells. More specifically, 231-M1 could express higher level of antioxidant proteins than 231-C3 cells. To identify

possible differentially expressed antioxidant proteins between these two cell lines, the protein profiling of 231-C3 and 231-M1 cells were compared using quantitative two-dimensional difference gel electrophoresis (2D-DIGE) proteomics in collaboration with Dr. Hongjin Huang from Applied Biomics (Hayward, CA, USA). Protein extracted from 231-C3 or 231-M1 cells was labeled with Cy2 (Ex492/Em510) or Cy3 (Ex550/Em570) dye respectively and was mixed together in equal amount. The protein mixture was subjected to 2D electrophoresis and separated as various individual protein spots on the gel. After fluorescence image scanning and image analysis, protein spots with different color were shown on the gel. Protein spot shows red color when protein contained in this spot was expressed with a higher level in 231-M1 than 231-C3, whereas green color spot represents the protein included in this spot has a higher expression level in 231-C3 than 231-M1. If one protein is expressed in a comparable level in both 231-C3 and 231-M1 cells, the spot of this protein shows yellow color (Figure 3-4).

Figure 3-4 Schematic diagram of 2D-DIGE proteomics. Protein extracted from 231-C3 or 231-M1 was labeled with Cy2 (Ex492/Em510) or Cy3 (Ex550/Em570) respectively. Protein expression ratio of 231-M1 to 231-C3 was calculated based on fluorescence intensity ratio of Cy3 to Cy2 emitted by each protein spot on the gel. Proteins of interest were further identified by LC-MS/MS.

Fluorescence images of analytical gel obtained in 2D-DIGE proteomics were shown in Figure 3-5.

Figure 3-5 Fluorescent images of analytical gel obtained in 2D-DIGE proteomics. The 2D gel was scanned using a Typhoon Trio scanner to obtain fluorescent images, which were further analyzed by Image Quant software. The fluorescence images taken at Ex492/Em510 of Cy2 and at Ex550/Em570 of Cy3 were overlapped and fluorescence intensity ratio of Cy3 to Cy2 was calculated to obtain protein expression ratio of 231-M1 to 231-C3.

54 protein spots with unequal fluorescence intensity of Cy2 and Cy3 were selected out (Figure 3-6) and proteins represented by these spots were identified through LC-MS/MS analysis. The assigned ID, protein expression ratio of 231-M1 to 231-C3 and protein identity for each of these 54 proteins were summarized in Table 3-1.

Figure 3-6 54 protein spots with unequal fluorescence intensity given by Cy2 and Cy3 were selected out. Proteins with higher expression level in 231-M1 compared to 231-C3 were circled in red, whereas the ones with lower expression level in 231-M1 compared to 231-C3 were circled in green.

Table 3-1 Information for the 54 differentially expressed proteins by 231-C3 and 231-M1

Assigned ID	Protein Expression Ratio	Protein Name
	231-M1 / 231-C3	
1	1.57	Protein-glutamine gamma-glutamyltransferase 2 OS=Homo sapiens GN=TGM2
2	2.11	Gelsolin OS=Homo sapiens GN=GSN
3	1.82	Moesin OS=Homo sapiens GN=MSN
4	2.29	Annexin A6 OS=Homo sapiens GN=ANXA6
5	1.68	Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2
6	-4.6	EH domain-containing protein 1 OS=Homo sapiens GN=EHD1
7	1.4	Pyruvate kinase PKM OS=Homo sapiens GN=PKM
8	-2.99	Heterogeneous nuclear ribonucleoprotein K OS=Homo sapiens GN=HNRNPK
9	1.4	Tubulin beta chain OS=Homo sapiens GN=TUBB
10	-3.83	Heterogeneous nuclear ribonucleoprotein H OS=Homo sapiens GN=HNRNPH1
11	1.6	Glucose-6-phosphate 1-dehydrogenase OS=Homo sapiens GN=G6PD
12	-2.7	Calumenin OS=Homo sapiens GN=CALU

13	-2.48	Eukaryotic initiation factor 4A-I OS=Homo sapiens GN=EIF4A1
14	-2.09	Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1
15	1.53	Alpha-enolase OS=Homo sapiens GN=ENO1
16	-2.99	Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1
17	-8.73	HLA class I histocompatibility antigen, A-2 alpha chain OS=Homo sapiens GN=HLA-A
18	-3.27	Heterogeneous nuclear ribonucleoprotein H OS=Homo sapiens GN=HNRNPH1 PE=1 SV=4 sapiens GN=HNRNPH1
19	-1.97	Heterogeneous nuclear ribonucleoproteins C1/C2 OS=Homo sapiens GN=HNRNPC
20	-3.61	Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB
21	-27.35	Protein PRR14L OS=Homo sapiens GN=PRR14L
22	-2.12	Coiled-coil domain-containing protein C16orf93 OS=Homo sapiens GN=C16orf93
23	-2.73	RNA-binding protein 4 OS=Homo sapiens GN=RBM4
24	2.27	Aspartate aminotransferase, cytoplasmic OS=Homo sapiens GN=GOT1
25	-7.09	Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB
26	-13.91	Tau-tubulin kinase 2 OS=Homo sapiens GN=TTBK2
27	-1.93	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit OS=Homo sapiens GN=PPP1CA
28	1.54	Annexin A1 OS=Homo sapiens GN=ANXA1
29	-2.86	Splicing factor U2AF 35 kDa subunit OS=Homo sapiens GN=U2AF1
30	-2.28	Malate dehydrogenase, cytoplasmic OS=Homo sapiens GN=MDH1
31	2.14	Microtubule-associated protein RP/EB family member 1 OS=Homo sapiens GN=MAPRE1
32	-4.12	Copper chaperone for superoxide dismutase OS=Homo sapiens GN=CCS
33	2.05	Annexin A4 OS=Homo sapiens GN=ANXA4
34	-4.74	E3 ubiquitin-protein ligase CHIP OS=Homo sapiens GN=STUB1
35	-3.83	Annexin A4 OS=Homo sapiens GN=ANXA4
36	1.74	Calretinin OS=Homo sapiens GN=CALB2
37	-1.59	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1
38	1.5	Glutathione S-transferase omega-1 OS=Homo sapiens GN=GSTO1
39	1.59	Rho GDP-dissociation inhibitor 1 OS=Homo sapiens GN=ARHGDIA
40	1.75	Rho GDP-dissociation inhibitor 2 OS=Homo sapiens GN=ARHGDIB
41	-4.59	COMM domain-containing protein 7 OS=Homo sapiens
42	-3.87	26S proteasome non-ATPase regulatory subunit 8 OS=Homo sapiens GN=PSMD8
43	1.46	Triosephosphate isomerase OS=Homo sapiens GN=TPI1
44	-2.33	GTP-binding nuclear protein Ran OS=Homo sapiens GN=RAN
45	-15.74	Triosephosphate isomerase OS=Homo sapiens GN=TPI1
46	1.4	Glutathione S-transferase P OS=Homo sapiens GN=GSTP1
47	-2.51	Glutathione S-transferase P OS=Homo sapiens GN=GSTP1
48	1.26	Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1
49	2.06	Hippocalcin-like protein 1 OS=Homo sapiens GN=HPCAL1
50	-4	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase OS=Homo sapiens GN=ADI1
51	1.57	Stathmin OS=Homo sapiens GN=STMN1
52	-3.38	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10
53	-3.34	Tubulin beta chain OS=Homo sapiens GN=TUBB
54	1.64	Protein S100-A11 OS=Homo sapiens GN=S100A11

In table 3-1, protein expression ratio was indicated by either positive value or negative value. Positive value means protein expression is up regulated in 231-M1 cells compared to 231-C3 cells, while negative value means protein expression is down regulated in 231-M1 cells compared to 231-C3 cells. Absolute value means the changing folds of protein expression. Cut-off of 1.3 was set up to define the up-regulation of protein expression in 231-M1 compared to 231-C3 (≥ 1.3) and down-regulation of protein expression in 231-M1 compared to 231-C3 (≤ -1.3) [141]. Protein expression ratio of -1.3 to 1.3 suggests protein is expressed in a comparable level in 231-C3 and 231-M1. As shown in Figure 3-7, there are total 22 proteins that are up regulated in 231-M1 cells and 31 proteins that are down regulated in 231-M1 cells compared to 231-C3 cells.

Figure 3-7 Grouping of identified proteins in terms of relative protein expression level

These identified 54 proteins were classified through online Panther classification system in terms of molecular function, protein class and biological process. I focused on three groups of proteins: proteins with antioxidant ability that can reduce oxidative stress directly (Figure 3-8A), the oxidoreductase that catalyzes redox

reactions in cells and thus probably facilitates the generation of intracellular antioxidant molecules (Figure 3-8B) and proteins with the function of response to stimulus as oxidative stress is one harmful stimulus to cells (Figure 3-8C).

Figure 3-8 Classification of total 54 differentially expressed proteins that were identified through 2D-DIGE proteomics by online Panther system. Three groups of proteins (antioxidant activity, oxidoreductase or response to stimulus) were selected.

There are total five proteins under these three groups. Of the five proteins, an oxidoreductase, G6PD is the only one that was significantly up regulated in 231-M1 cells compared with 231-C3 cells by 1.6 folds. All other four proteins were down regulated from 231-C3 to 231-M1 (Table 3-2).

Table 3-2 Information of 5 proteins selected by Panther classification system

Assigned ID	Protein expression ratio 231-M1 / 231-C3	Protein name	Protein function
11	1.6	Glucose-6-phosphate 1-dehydrogenase (G6PD)	Oxidoreductase
27	-1.9	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PPP1CA)	Response to stimulus
30	-2.3	Malate dehydrogenase, cytoplasmic (MDH1)	Oxidoreductase
32	-4.1	Copper chaperone for superoxide dismutase (CCS)	Antioxidant activity Oxidoreductase Response to stimulus
50	-4.0	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase (ADI1)	Oxidoreductase

In summary, G6PD was selected out from total 54 identified proteins for further analysis. First, G6PD is a cellular oxidoreductase that is able to catalyze redox reactions in cells and thus probably facilitates the generation of intracellular antioxidant molecules. Second, G6PD is the only oxidoreductase that was found to up-regulate in 231-M1 cells compared to 231-C3 cells by this run of 2D-DIGE proteomics study. Although the G6PD expression change ratio is not amongst the highest change ratio of all identified proteins, its potential antioxidant function is most closely linked to the objective of this proteomics study, which is to discover antioxidant proteins that is up-regulated in 231-M1 cells over 231-C3 cells. Therefore, G6PD was selected out and subjected to further analysis.

The PPP1CA is one of the three catalytic subunits of protein phosphatase 1 (PP1) that is known to participate in several cellular processes, such as muscle contractility, cell division and glycogen. This aforementioned function of PP1 is not closely linked to cell antioxidant ability. MDH1 catalyzes the reversible oxidation of malate to oxaloacetate, during which one reducing agent NADH is formed. CCS is a metalloprotein with the function of delivering Cu to superoxide dismutase (SOD 1). SOD1, located in cytoplasm and mitochondrial intermembrane space, is one of three superoxide dismutases. It plays pivotal role in destroying free superoxide radicals. Both MDH1 and CCS have some antioxidant ability in terms of

their respective functions. However, both of these two enzymes are down regulated in 231-M1 cells compared to 231-C3 cells. ADI1 catalyzes the formation of formate and 2-keto-4-methylthiobutyrate, during which no reducing agent is generated.

The assigned ID for G6PD was 11 and it was shown on the gel as an orange color spot, which means 231-M1 cells have higher G6PD expression level than 231-C3 cells (Figure 3-9).

Figure 3-9 Fluorescent image of 2D-DIGE proteomics gel. G6PD was assigned with ID 11 and was shown as orange spot on the gel that was enlarged in the upper right corner of the image.

The up-regulation of G6PD by proteomics result was validated by western blot analysis on 231-C3 and 231-M1 cells. The quantified ratio of G6PD in 231-M1 to 231-C3 cells was 1.6 in proteomics and 3.2 in Western blot result (Figure 3-10).

Figure 3-10 The up-regulation of G6PD by proteomics result was validated by western blot analysis. (A) Western blot analysis of the G6PD and β -actin levels in 231-C3 and 231-M1 cells. (B) The quantified ratio of G6PD in 231-M1 to 231-C3 cells in proteomics and western blot result.

3.3 Discussion

Various experimental approaches were applied by researchers to explore the mechanisms of anti-cancer drug resistance. Most studies focused on drug treatment-induced chemotherapeutic agents resistance *in vitro* or in mice xenografts that do not involve the metastasis process of cancer cells [142, 143] or comparison of tumor samples obtained pretreatment, on-treatment or after relapse with normal samples from cancer patients that possess a high clinical relevance but require many months or years to complete sample collection [144]. Our model simulates the physiological process of metastasis, focusing on the late stages of metastasis during which cancer cells adapt to environment at the metastatic site and survive, and thus offers the opportunity to explore the mechanisms of drug resistance acquired through metastasis process without the interference from anti-cancer drug selection effect. Furthermore, this study is closely relevant to the clinical situation that many cancer patients have not been diagnosed with breast cancer until late stage when tumor cells already spread out to distant organs and these tumors respond poorly to the anthracycline-based chemotherapy [145, 146].

There are many mechanisms underlying DOX resistance of cancer cells that have been reported, which include up-regulation of multi-drug resistance protein 1 (MDR1; also known as P-glycoprotein), down-regulation or mutation of topoisomerase II, decreased apoptosis caused by p53 mutation and altered proliferation of cancer cells [147, 148]. Although oxidative stress generation ability of DOX has been intensively studied [45-48], there are few research works that explains DOX resistance of cancer cells in terms of their antioxidant ability.

It was suggested that enhanced glutathione peroxidase activity and its increased expression level in DOX-resistant cells protect cells from DOX-induced lipid peroxidation and hence could lead to cell resistance to DOX [74]. A similar finding was reported by another study that glutathione peroxidase detoxifies oxygen radicals induced by DOX and hence contributes to DOX resistance of sarcoma cell lines derived from clinical samples [75]. More directly, load MCF-7 cells with glutathione peroxidase or superoxide dismutase can generate DOX resistance in cells [76].

A previous study in our lab successfully demonstrated that knockdown of MnSOD, a mitochondrial antioxidant protein, significantly sensitized TNBC cells to DOX induced apoptosis by overcoming the protection of cells exerted by MnSOD from superoxide (one kind of ROS) induced mitochondrial damage [14].

Inspired by these previous studies, in current research work, I attempted to explore other possible antioxidant proteins that protect TNBC cells from oxidative stress induced by DOX and hence lead to DOX resistance and to discover possible therapeutic targets for mitigating DOX resistance.

3.4 Conclusion

In this chapter, the generation process of metastatic TNBC cell line, 231-M1 is introduced. 231-C3 cells were injected into tail vein of a nude mouse. After 45 days, a micro-metastasis on the lung of the mouse was dissected for primary culture and a novel lung metastatic TNBC cell line, 231-M1 was generated. Upon DOX treatment that was demonstrated to produce high level of intracellular oxidative stress in this chapter, metastatic 231-M1 cells displayed significantly higher cell viability and lower apoptotic rate than their parental 231-C3 cells. Through protein profiles comparison between 231-C3 and 231-M1 by 2D-DIGE proteomics study, G6PD, an important antioxidant protein existing in cytoplasm was demonstrated to up regulate in 231-M1 cells compared with 231-C3 cells. The up regulation of G6PD was further validated by Western blot analysis.

Chapter 4 G6PD Mediates Metastasis-acquired and Intrinsic Doxorubicin Resistance of Triple Negative Breast Cancer Cells by Maintaining GSH Level

4.1 Introduction

In Chapter 3, glucose-6-phosphate dehydrogenase (G6PD) was identified to up regulate in metastatic compared to non-metastatic TNBC cells in 2D-DIGE proteomics. Cancer cells are known to develop increased antioxidant ability to avoid damage effect caused by either endogenous or exogenous ROS, such as oxidative stress induced by anti-cancer drugs [149]. The production of glutathione (GSH), one of the most abundant intracellular antioxidants, has been shown to be up-regulated in cancer cells [120]. The oxidative branch of pentose phosphate pathway (PPP) is a major source of nicotinamide adenine dinucleotide phosphate (NADPH), which is essential for generating intracellular GSH [150]. G6PD that catalyzes the first step of oxidative branch of PPP is the rate-limiting enzyme of this metabolic pathway. The expression level of G6PD has been reported to increase in various cancer lesions, especially in cancers with advanced stages and metastatic disease, which is associated with poor treatment outcome [130-133]. In this chapter, the role of G6PD in DOX resistance of TNBC cells was investigated through exploring clinical database and *in vitro* studies.

4.2 Results

4.2.1. High G6PD Expression is Correlated with Poor DOX Response in TNBC Cells

The G6PD expression level was examined by western blot analysis in the human mammary epithelial cell line MCF10A and four TNBC cell lines including MDA-MB-157, BT549, MDA-MB-231 and MDA-MB-468. These cells were treated with DOX at various concentrations for 24 h and the IC_{50} of DOX in each cell line was calculated with GraphPad. The result shows the higher the G6PD expression level in cells, the lower sensitivity of cells towards DOX treatment. In particular, MCF10A cells which have a very low level of G6PD were sensitive to a DOX concentration of $3.5 \pm 1.3 \mu M$, while high G6PD expressing MDA-MB-468 cells were tolerant to DOX over $10 \mu M$ (Figure 4-1A). Linear regression analysis revealed a strong positive correlation between the G6PD level and DOX IC_{50} with a R-square of 0.993 (Figure 4-1B).

Figure 4-1 High G6PD expression is positively correlated with poor DOX response in TNBC cells. (A) Upper panel shows the western blot analysis of G6PD level in MCF10A and four TNBC cell lines including MDA-MB-157, BT549, MDA-MB-231 and MDA-MB-468.

Lower panel shows IC₅₀ of DOX in five cell lines: MCF10A, MDA-MB-157, BT549, MDA-MB-231 and MDA-MB-468, which was calculated from MTT assay results. (B) Linear regression analysis of G6PD expression level and DOX IC₅₀ of these cell lines revealed a strong positive correlation with a R-square of 0.993.

4.2.2. High G6PD Expression is Associated with Low Overall Survival Rate of TNBC Patients

Analysis of Curtis et al. (2012) breast cancer dataset [136] revealed that the average G6PD expression level in TNBC is higher than in normal tissue (Welch's t-test, p value $< 2.2 \times 10^{-16}$) (Figure 4-2A), which further indicated a functional importance of G6PD in TNBC cell survival. Moreover, the overall survival analysis revealed that TNBC patients with higher G6PD expression tended to have lower survival probability compared to those with relatively low G6PD expression (Figure 4-2B). Given that most of cancer patients received DOX for cancer treatment and survival period extension, the relatively low survival rate of patients with high G6PD expression supports the hypothesis that G6PD participates in DOX resistance of TNBC and may lead to treatment failure.

Figure 4-2 Clinical data analysis (A) The comparison of G6PD expression level between TNBC and normal tissues. Data is taken from Curtis et al (2012), and sample size is indicated in each type. (B) The overall survival probabilities of TNBC patients with high and low G6PD expression in Curtis et al (2012). P-value was calculated using Mantel-Haenszel logrank test.

4.2.3. Down-regulating G6PD Expression Reduces Cell Viability and Increases Apoptotic Rate of TNBC Cells upon DOX Treatment

To demonstrate G6PD mediates DOX resistance of metastatic TNBC cells, the specific G6PD siRNA was applied to suppress G6PD expression inside cells, which was followed by functional analysis. Western blot analysis showed G6PD expression was successfully silenced by specific siRNA in both 231-C3 and 231-M1 cells (Figure 4-3).

Figure 4-3 Western blot analysis of G6PD levels in 231-C3 and 231-M1 cells that were without siRNA transfection or transfected with 20 nM of siNeg or siG6PD.

Cells transfected with negative control siRNA or siG6PD or without siRNA transfection were treated with DOX at 2.5 μ M, 5 μ M and 10 μ M for 24 h. MTT results show cells with G6PD knockdown exhibited significantly lower cell viability after 24 h of DOX treatment compared with cells in control and siNeg group. Specifically, after 24 h of DOX treatment at 2.5 μ M, 5 μ M and 10 μ M, the relative cell viability of 231-C3 cells with G6PD silencing is 30%, 19% and 18% respectively, which was much less than that of siNeg group (62%, 43% and 38% respectively) (Figure 4-4A). The similar results were also obtained in metastatic 231-M1 cell line, despite their higher G6PD level. After 24 h of DOX treatment at 5 μ M and 10 μ M, the G6PD silenced cells exhibited a 31% cell viability, which

was almost the half of cell viability in siNeg transfected cells (61% at 5 μ M and 59% at 10 μ M respectively) (Figure 4-4B). Overall, the MTT assay results showed reduction of G6PD expression in either metastatic TNBC cells 231-M1 or original 231-C3 cells leads to increase of DOX effectiveness on these cells.

Figure 4-4 G6PD silencing by siRNA decreased cell viability of both 231-C3 and 231-M1 cells upon DOX treatment. (A) and (B) show the MTT assay results of 231-C3 and 231-M1 cells respectively. After 45 h post siRNA transfection, the cells were treated with DOX at 2.5 μ M, 5 μ M and 10 μ M respectively for 24 h. The results were normalized to the control group without DOX treatment.

The decreased cell viability after G6PD silencing was obviously due to the increased apoptosis induced by DOX. As shown by the FRET images, at 24 h of DOX treatment, more apoptotic cells were observed in 231-M1 cells with siG6PD treatment in all three concentrations (2.5 μ M, 5 μ M and 10 μ M), while more apoptotic cells were detected in siG6PD treated 231-C3 cells under 2.5 μ M DOX treatment (Figure 4-5A). In 231-C3 cells treated with 5 μ M and 10 μ M DOX, apoptotic rates were already very high (above 50%) in control and siNeg transfected cells, silencing G6PD further increased about 20% of apoptosis. Specifically for 231-M1 cells, without silencing G6PD, only around 30% of apoptosis was observed in highest DOX concentration of 10 μ M. After G6PD silencing, the apoptotic rate increased to 45%, 63% and 69% for 24 h of 2.5 μ M, 5 μ M and 10 μ M DOX

treatment respectively, which was around 2-fold increase for all conditions (Figure 4-5B).

Figure 4-5 G6PD silencing by siRNA increased apoptotic rates of both 231-C3 and 231-M1 cells. (A) Representative FRET images of 231-C3 and 231-M1 cells with either siNeg or siG6PD treatment that were further subjected to DOX treatment at 0 μ M, 2.5 μ M, 5 μ M and 10 μ M respectively for 24 h. Scale bar, 100 μ m. (B) Apoptotic rates of 231-C3 and 231-M1 cells that were quantified from FRET images.

The results were also validated with wild type MDA-MB-231 cells and another TNBC cell line, MDA-MB-468. Silencing G6PD successfully reduced the overall level of G6PD in these two TNBC cell lines (Figure 4-6).

Figure 4-6 Western blot analysis of G6PD levels in MDA-MB-231 and MDA-MB-468 cells that were transfected with 20 nM of either siNeg or siG6PD.

After G6PD knockdown, the cells were further subjected to DOX treatment and MTT assay. Results showed the G6PD reduction in MDA-MB-231 cells decreased cell viability to 40% after 24 h and 9% after 48 h of 5 μ M DOX treatment from 70%

and 27% with normally G6PD expressed cells (Figure 4-7A). In MDA-MB-468 cells, the viability decreasing induced by G6PD reduction is 25% after 24 h and 20% after 48h of 5 μ M DOX treatment (Figure 4-7B). Notably, for both MDA-MB-231 and MDA-MB-468 cells treated with DOX for 48 h, the cell viability of G6PD silencing cells was less than half of control cells.

Figure 4-7 G6PD silencing by siRNA decreased cell viability of both MDA-MB-231 and MDA-MB-468 cells upon DOX treatment. (A) and (B) show the MTT assay results of MDA-MB-231 and MDA-MB-468 cells respectively. After 45 h post siRNA transfection, the cells were treated with DOX at 5 μ M for either 24 h or 48 h before subjecting to MTT assay. The results were normalized to the control group without DOX treatment.

4.2.4. Inhibiting G6PD Activity with Its Inhibitor DHEA Sensitize TNBC Cells to DOX Treatment

To further demonstrate G6PD plays an important role in DOX resistance of metastatic and original TNBC cells and to show G6PD has the potential to serve as a druggable target for breast cancer treatment, the G6PD activity of cells was inhibited with its chemical inhibitor dehydroepiandrosterone (DHEA) [118, 151-153]. DHEA is a well-known uncompetitive G6PD inhibitor, which exerts its inhibition effect by binding to G6PD ternary enzyme-substrate complex [151]. With G6PD inhibition, the cellular response towards DOX treatment was investigated. 231-C3 and 231-M1 cells were pretreated with 200 μ M of DHEA for 12 h to inhibit

the G6PD activity. After that, the cells were treated with both of 200 μ M DHEA and DOX at 1.25 μ M, 2.5 μ M and 5 μ M respectively for another 24 h or 48 h. Compared with cells treated with DOX alone, cells that were treated with DHEA and DOX together exhibited significantly lower cell viability examined by MTT assay (Figure 4-8). In particular, when G6PD was inhibited by DHEA, the cell viability of 231-C3 cells reduced from around 80%, 60% and 45% to around 20% after 24 h of DOX treatment at 1.25, 2.5, 5 μ M respectively (Figure 4-8A). The viability of 231-M1 cells also showed a 40% reduction when they were treated with DOX and 200 μ M DHEA together (Figure 4-8B). Very interestingly, when the treatment time extended to 48 h, the cell viability of 231-C3 was dramatically decreased to around 5% and that of 231-M1 was also decreased to around 10% in all three DOX concentrations when G6PD was inhibited with DHEA, which indicates the vast majority of cells were dead (Figure 4-8C and Figure 4-8D). Notably, when cells were treated with 200 μ M DHEA alone for overall 36 h or 60 h, the cell viabilities of both 231-C3 and 231-M1 cells were above 80% and above 50% respectively, suggesting cell toxicity induced by DHEA alone is not big (Figure 4-8).

Figure 4-8 231-C3 or 231-M1 cells were pretreated with or without 200 μ M DHEA for 12 h and then cells were subjected to combinational treatment of 200 μ M DHEA with DOX at 1.25 μ M, 2.5 μ M or 5 μ M respectively or DOX treatment alone for a further 24 h or 48 h. After treatment, cell viability was determined by MTT assay and normalized to cells without

any treatment. The results were shown as 231-C3 24h (A), 231-M1 24h (B), 231-C3 48h (C) and 231-M1 48h (D).

The apoptotic rates of 231-C3 and 231-M1 cells after 24 h of DOX treatment with or without G6PD inhibition by 200 μ M of DHEA were revealed by FRET imaging. DHEA alone at a concentration of 200 μ M did not induce any obvious apoptosis in both 231-C3 and 231-M1 cells. G6PD inhibited cells exhibited significantly increased cell apoptosis under DOX treatment compared to cells without G6PD inhibition, which was reflected by a larger proportion of blue cells (cell undergoing apoptosis) shown in FRET images of DHEA treatment group compared with W/O DHEA treatment group (Figure 4-9).

Figure 4-9 Representative FRET images of 231-C3 and 231-M1 cells with DHEA treatment alone, with DOX treatment alone, with combinational treatment or without any treatment.

The apoptotic rates quantified from FRET image revealed pre-treatment with 200 μ M of DHEA for 12 h to inhibit G6PD activity significantly sensitized 231-C3 and

231-M1 cells to DOX-induced apoptosis. In particular, when 231-C3 cells were treated with DOX alone at 1.25 μ M, 2.5 μ M and 5 μ M for 24 h, the apoptotic rates were around 0%, 20% and 40% respectively. In contrast, G6PD inhibition by DHEA increased the apoptotic rates to above 50% upon 24 h of DOX treatment at all three DOX concentrations (Figure 4-10A). In 231-M1 cells, the apoptotic rates with DOX treatment alone at 1.25 μ M, 2.5 μ M and 5 μ M were 2%, 10% and 23% respectively, which increased to around 40% at all three DOX concentrations when combining DHEA treatment (Figure 4-10B). It is notable that there was almost no apoptosis detected when 231-C3 and 231-M1 cells were treated with 1.25 μ M DOX alone, while the apoptotic rates were increased dramatically to 54% and 40% for 231-C3 and 231-M1 cells respectively upon combining DOX treatment with 200 μ M of DHEA treatment, which indicates G6PD inhibition increased DOX effectiveness on metastatic and original TNBC cells to a great extent (Figure 4-10).

Figure 4-10 Apoptotic rates of 231-C3 and 231-M1 cells that were quantified from FRET images. 231-C3 or 231-M1 cells with or without 12 h of 200 μ M DHEA pretreatment were subjected to combinational treatment of 200 μ M DHEA with DOX at 1.25 μ M, 2.5 μ M or 5 μ M respectively or DOX treatment alone for a further 24 h.

The similar results were also obtained in wild-type MDA-MB-231 and MDA-MB-468 cells. Combinational treatment with DHEA and DOX significantly reduced cell viability of MDA-MB-231 (Figure 4-11A) and MDA-MB-468 cells (Figure 4-11B). In 24 h of DOX treatment, when G6PD activity was inhibited by DHEA, cell viability was reduced to less than half of cells without G6PD inhibition at the same DOX concentration (Figure 4-11). In 48 h of combinational treatment, the viability of MDA-MB-231 cells was reduced to less than 10% at all three DOX concentrations, which was in sharp contrast to the cell viability in DOX treatment alone (above 30% at 1.25 μ M) (Figure 4-11A). In MDA-MB-468 cells, combination of DHEA with 1.25, 2.5 and 5 μ M DOX also decreased cell viability

from around 30% to around 10% in all three DOX concentrations. These results indicated a high pharmaceutical potential of G6PD inhibitor in treating DOX resistant TNBC tumor.

Figure 4-11 (A) MTT assay result of MDA-MB-231 cells with or without 12 h of 200 μM DHEA pretreatment before subjecting to combinational treatment of 200 μM DHEA with DOX (1.25 μM, 2.5 μM or 5 μM respectively) or DOX treatment alone for a further 24 h or 48 h. The results were normalized to the control group without any treatment. (B) MTT assay result of MDA-MB-468 cells with or without 12 h of 200 μM DHEA pretreatment before subjecting to combinational treatment of 200 μM DHEA with DOX (1.25 μM, 2.5 μM or 5 μM respectively) or DOX treatment alone for a further 24 h or 48 h. The results were normalized to the control group without any treatment.

4.2.5. G6PD Enables TNBC Cells to Maintain a Relatively High Intracellular GSH Level during DOX Treatment

I was then interested in the mechanism of G6PD mediated DOX-resistance. Intracellular DOX concentration was first measured to check whether the differential DOX response between 231-C3 and 231-M1 cells was due to differential drug uptake or multidrug efflux pumps. When cells were treated with DOX at 5 μ M, the intracellular DOX concentration of 231-C3 and 231-M1 cells was detected and calculated based on the red fluorescence emitted from DOX. The result shows during 24 h of DOX treatment, DOX was accumulated inside cells gradually. The intracellular DOX concentrations during 24 h of DOX treatment were similar between 231-C3 and 231-M1 cells, which excludes the decreased drug uptake as major mechanism of DOX resistance in metastatic 231-M1 cells (Figure 4-12).

Figure 4-12 Intracellular DOX concentration of 231-C3 and 231-M1 cells upon 5 μ M DOX treatment for 0, 1, 3, 6, 10 and 24 h respectively. Total DOX amount inside cells was normalized to total protein amount of the cells.

As the rate-limiting enzyme of PPP, one major function of G6PD is to provide reducing molecule NADPH for cellular antioxidant defense, which further quickly reacts with glutathione disulfide (GSSG) to generate the reduced GSH under the catalysis of glutathione reductase (GSR) [126]. GSH is a major antioxidant molecule inside cells and can protect cells from apoptosis induced by ROS generation of DOX [154-156]. Based on this knowledge, it was hypothesized that the promotion of GSH generation exerted by G6PD is the most possible mechanism underlying its protection of cancer cells from oxidative stress induced by DOX.

Following aforementioned hypothesis, the GSH level was measured in 231-C3 and 231-M1 cells during 5 μ M DOX treatment for up to 48 h. The result of 231-C3 cells shows that DOX treatment could exhaust the GSH pool after 24 h by decreasing the reduced GSH level (Figure 4-13). In contrast, the reduced GSH level in 231-M1 cells was relatively stable over DOX treatment compared to 231-C3 cells (Figure 4-13). In particular, DOX treatment reduced the intracellular GSH level of 24% and 47% in 231-C3 cells after 24 h and 48 h of treatment respectively, which was consistent with the viability decrease of the cells. In 231-M1 cells, the GSH reduction was only 13% after 24 h and 23% after 48 h of DOX treatment, which was consistent with a relatively higher viability and lower apoptotic rate of the cells compared to 231-C3 counterpart (Figure 4-13).

Figure 4-13 Relative reduced GSH level inside 231-C3 and 231-M1 cells upon 5 μ M DOX treatment at 0, 5, 10, 24 and 48 h respectively. GSH levels were normalized to GSH level at 0 h of DOX treatment.

Reducing G6PD level by siRNA transfection further promotes the exhaustion of the reduced GSH pool in 231-C3 cells and also abrogated the relatively stable GSH level in 231-M1 cells. As shown in Figure 4-14A, when G6PD expression was suppressed, the intracellular GSH level of 231-C3 cells was reduced from 77% to 57% after 24 h DOX treatment at 5 μ M. DOX treatment also reduced the GSH level in 231-M1 cells for a further 20% in G6PD silencing cells compared to normal cells after 24 h of 5 μ M DOX treatment (Figure 4-14B). Overall, the results suggest G6PD enable TNBC cells to maintain a relatively stable intracellular GSH level during DOX treatment.

Figure 4-14 (A) 231-C3 cells were transfected with either negative or G6PD specific siRNA before DOX treatment. GSH level inside 231-C3 cells upon 5 μ M DOX treatment at 0, 12, and 24 h respectively was measured and normalized to GSH level at 0 h of DOX treatment. (B) Relative GSH level inside 231-M1 cells upon 5 μ M DOX treatment at 0, 12, and 24 h respectively after 231-M1 cells were transfected with either negative or G6PD specific siRNA. GSH levels were normalized to GSH level at 0 h of DOX treatment.

4.3 Discussion

The results in this chapter shows either reduction of G6PD expression by siRNA or inhibition of its activity by specific inhibitor DHEA successfully sensitized TNBC cells, especially the cells derived from lung metastasis, to DOX treatment.

The Warburg effect that most cancer cells rely on aerobic glycolysis even in the presence of sufficient oxygen to generate necessary energy has been widely accepted for several decades [108]. The PPP that branches from glycolysis has attracted more and more attentions in recent years as this metabolic pathway does not only satisfy cancer cells' anabolic requirements but also plays an essential role to generate intracellular antioxidants that enable glycolytic cancer cells to combat oxidative stress [109, 112]. Considering the fundamental role PPP plays to support cancer cell survival and the “gatekeeper” role the G6PD plays in PPP by directing glucose metabolite into PPP and hence controlling the whole efficiency of this pathway, G6PD has attracted many interests by serving as a potential therapeutic

target for cancer treatment. It was reported that G6PD protects cells from apoptosis induced by ionizing radiation through maintaining intracellular GSH level [157]. DOX resistant cancer cells exhibit higher G6PD activity and higher level of reduced GSH compared with parental drug-sensitive cells [158-160]. Consistent with these observations, this study found G6PD was up regulated in metastatic TNBC cells that acquire DOX resistance after metastasis. More importantly, the study in this chapter demonstrated for the first time that suppression of G6PD abrogated DOX resistance of metastatic TNBC cells, and also increased sensitivity of original TNBC cells to DOX treatment.

In another research work, the DOX-resistant human colon cancer cells that show increased activity of PPP and G6PD have lower intracellular DOX accumulation in comparison with DOX-sensitive counterpart. The author concluded that high GSH level helps cells to extrude DOX out of cells [57]. Here I measured DOX concentration in metastatic and parental cells and found there is no significant difference of DOX uptake between these two cells lines and exclude the possibility of drug pumps mediating DOX resistance acquired after metastasis. Upon DOX treatment, the metastatic TNBC cells that maintain higher GSH level exhibited lower apoptosis rate compared to parental cells. When G6PD expression was suppressed, the apoptotic rate of cells increased, accompanied by decreased intracellular GSH level. The important apoptosis mediator, cytochrome c was shown to keep in reduced and inactive form by intracellular GSH [161-163]. Therefore, I concluded stable GSH level maintained by G6PD even with oxidative stress exerted by DOX enable cells to become insensitive to apoptosis and thus conferred the metastatic cells with DOX resistance.

Furthermore, the uncompetitive G6PD inhibitor DHEA [151] successfully reversed intrinsic and metastasis-acquired DOX resistance of TNBC cells. Unfortunately, high lipid solubility of DHEA as well as few availability of G6PD inhibitors limits the investigation of its *in vivo* use [164].

4.4 Conclusion

In summary, the in-vitro studies showed that the higher level of G6PD the TNBC cells express, the poorer DOX response the cells exhibit. Through clinical database analysis, the high G6PD expression TNBC patients display lower survival rate than relatively low G6PD expression patients, suggesting that high G6PD expression confers TNBC with DOX resistance and hence results in poor treatment outcome. Either reducing G6PD expression by its specific siRNA or decreasing its activity by its inhibitor DHEA, leads to significantly decreased cell viability and increased apoptotic rate of metastatic TNBC cells as well as original ones upon DOX treatment. Through these studies, G6PD is demonstrated to mediate metastasis acquired and intrinsic DOX resistance of TNBC cells. Therefore, G6PD is a potential therapeutic target for sensitizing TNBC towards DOX treatment.

Chapter 5 Establishment of Caspase-3 Activation as Direct Measurement and Early Indicator for Hematologic Toxicity of Chemotherapy

5.1 Introduction

Chemotherapy is an indispensable part of the multidisciplinary treatment of breast cancer. An estimated two-thirds of women diagnosed with breast cancer receive chemotherapy. Anthracyclines are part of many of the standard chemotherapy regimens recommended by the NCCN and ASCO guidelines, which is associated with acute dose-limiting hematologic toxicity. Besides the decreased drug efficacy caused by drug resistance developed by breast cancer cells, hematologic toxicity is also closely associated with failure of anthracyclines treatment and are an inevitable consequence of non-selective effects of anti-cancer drugs on both cancerous and healthy cells. Among hematologic toxicity that is most common type of toxicities induced by anti-cancer drugs, neutropenia is a potentially serious side effect of several commonly used chemotherapy regimens and predisposes cancer patients to life-threatening infections, which are reportedly more frequent in Asian women [165]. It is dose-dependent, but sensitivity to myelotoxicity varies among individuals and there is currently no reliable predictor of hematologic toxicity. Because doses are determined based on a 'standard'

guideline, dose adjustments are usually needed and made only when patients manifest side effects. By the time a fall in white blood cell (WBC) count is detected, little can be done other than administering granulocyte-colony stimulating factor (G-CSF) to boost WBC production and empirical antibiotics if fever is also present. This is less than satisfactory. While it would be ideal to determine the appropriate dose of anticancer drug prior to treatment commencement, this is still not possible given existing knowledge and technologies.

The objective of this chapter is to develop a direct and simple method to assess hematologic toxicity in cancer patients receiving chemotherapy and to potentially predict hematologic adverse event at early stage of treatment regimen. In this way, this measurement is potentially able to guide dose modification and supportive care application in breast cancer patients receiving chemotherapy, especially anthracycline-containing ones.

I hypothesized that the level of active capase-3 present in blood plasma of cancer patients can measure the level of hematologic toxicity at an early stage of the chemotherapy regimen. My idea was formed on the basis that most of the anti-cancer drugs, including anthracyclines, induce cell death through apoptosis. During cell apoptosis, capase-3 is activated [166]. Since anti-cancer drugs are usually given via intravenous injection, majority of body cells are subjected to the drug's toxic effect and many non-cancerous cells also go apoptosis during chemotherapy, especially the fast-dividing cells, such as hematopoietic cells in bone marrow. Under normal circumstances, during cell apoptosis, apoptotic bodies form and are engulfed by phagocytes that are composed of many kinds of white blood cells derived from stem cells in the bone marrow and present in the bloodstream. Therefore, intracellular content of apoptotic cells will not be released into blood and

thus a harmful inflammatory response is avoided [167]. However, when the extent of bone marrow suppression and white blood cell death induced by chemotherapy drugs overwhelms the body's innate ability to remove apoptotic bodies, apoptotic bodies burst and contents inside them are released into the bloodstream, resulting in cellular damage.

Caspase-3 is usually present in the cells as an inactive pro-enzyme. It is only activated during apoptosis [166, 168]. Even then, caspase-3 shall be retained within apoptotic cells without being released to the extracellular environment. I predicted that caspase-3 will be released into the extracellular environment and enter blood stream when the white blood cells are severely damaged and the number of them is largely reduced due to bone marrow suppression (hematologic toxicity of anti-cancer drugs) during chemotherapy. Thus, elevated level of active caspase-3 in the blood plasma of cancer patients is potentially to provide a more direct measure of hematologic toxicity induced by chemotherapy on cancer patients and may also predict the development of neutropenia after cumulative doses at the early stage of chemotherapy regimen.

5.2 Results

5.2.1. Optimization of Caspase-3 Assay

Previously, caspase-3 assay developed by our group was used to detect caspase-3 activation inside cells that undergo apoptosis. In current study, however, the sample is blood plasma, and I do not have previous knowledge about how the components of blood plasma will affect the sensitivity of caspase-3 assay. The components of

blood plasma include 92% water, 8% blood plasma proteins and trace amount of other materials. I doubted that this high concentration of plasma proteins would prevent caspase-3 from reacting with its substrate by hindering them from contacting each other or these plasma proteins would block or absorb the fluorescence emitted by cleaved substrate.

Caspase-3 assay has been developed for detecting low concentration of caspase-3 in blood plasma. The aim of the first experiment is to find out the effect of plasma concentration on sensitivity of caspase-3 assay. Different amount of fetal bovine serum (FBS) was added in reaction mixture as substitute of blood plasma to achieve the final concentration of 5%, 10%, 20%, 40%, and 80% respectively. Activated caspase-3 was contained in cell lysate from different number of HeLa cells undergoing apoptosis after UV radiation. The result is shown in Figure 5-1:

Figure 5-1 Effect of plasma concentration on sensitivity of caspase-3 assay. Cell lysate from different amount of HeLa cells undergoing apoptosis after UV radiation was incubated with caspase-3 substrate for 24 h. The reaction mixture contains various concentrations of FBS.

When the concentration of plasma is high, such as 40% and 80%, there is no signal can be detected. When the concentration is reduced to 20%, relatively low intensity of fluorescence can be detected by spectrofluorometer. The fluorescence

intensity increases along with the decrease of plasma concentration. This result suggests high concentration of plasma interfere with reaction of caspase-3 with its substrate or the plasma proteins block or absorb the fluorescence emitted by cleaved substrate. However, the concentration of caspase-3 also decreases along with the dilution of sample, which may lead to unsuccessful caspase-3 detection. Based on this observation and consideration, I decided to add plasma sample in reaction mixture at final concentration of 10% for caspase-3 assay.

It was further found that both the plasma and original un-cleaved substrate give auto-fluorescence that could interfere with the result of caspase-3 assay. The result is shown in Figure 5-2.

Figure 5-2 Auto-fluorescence of plasma and substrate. Various amount of plasma (A) or various amount of original substrate (B) was mixed with reaction buffer of caspase-3 assay respectively. Fluorescence was detected at different time points (4 h, 6 h, 8 h, 24 h, 48 h and 72 h).

Due to auto-fluorescence emitted by original substrate that can influence the accuracy of caspase-3 assay, the amount of substrate used for caspase-3 assay needs to be minimized. However, if substrate concentration is too low, the reaction between caspase-3 and its substrate may not be efficient due to the insufficient contact between caspase-3 and its substrate resulted from low concentration of both caspase-3 inside sample and substrate. Thus, an optimal amount of substrate needs

to be added into reaction mixture to minimize the background fluorescence as well as ensure efficiency of reaction. The result shown in Figure 5-3 indicates 0.5 μ l of substrate is the most suitable amount to be added into reaction system for active caspase-3 detection.

Figure 5-3 The effect of different amounts of substrate added in reaction mixture on reaction efficiency of caspase-3 with its substrate. 1 μ l of cell lysate containing active caspase-3 from apoptotic HeLa cells was mixed with various amount of substrate. The fluorescence was detected at different reaction time points (4 h, 6 h, 8 h, 24 h, 48 h and 72 h).

In addition to plasma sample and substrate added in reaction mixture, the reaction buffer for caspase-3 assay also contains 20 mM Hepes-KOH pH=7.5 to maintain the optimal pH for enzyme-substrate reaction, 2 mM EDTA and 1 $\times$ protease inhibitor to protect caspase-3 from being degraded by proteases, and 1 mM DTT to maintain a reducing environment that simulate the intracellular conditions for enzyme-substrate reaction. The finalized formula for reaction mixture of caspase-3 assay is shown in Table 5-1.

Table 5-1 Finalized formula for reaction mixture of caspase-3 assay

Sample and Reagent	Volume	Final Concentration
Plasma	25 μ l	10%
200 mM Hepes-KOH pH=7.5	25 μ l	20 mM
100 mM EDTA	5 μ l	2 mM
100 mM DTT	2.5 μ l	1 mM
100 $\times$ Protease inhibitor	2.5 μ l	1 $\times$
Ac-DEVD-AMC	0.5 μ l	0.2%
DI water	190 μ l	76%
Total volume	250 μ l	

5.2.2. Preclinical Study

In preliminary studies, human leukemia cells K562 were treated with anti-cancer drugs, DOX and paclitaxel respectively, and increased caspase-3 activity levels in culture media were detected by caspase-3 assay. Similarly, increased caspase-3 level was also detected in culture media of porcine white blood cells after treated with paclitaxel.

The result shows that both doxorubicin and paclitaxel activated caspase-3 in leukemia K-562 cells, and the caspase-3 enzyme was released from cells into the culture medium and was successfully detected using caspase-3 assay (Figure 5-4). In addition to cancer cells, paclitaxel also induced the while blood cells from pig blood into apoptosis, and the caspase-3 released from the porcine white blood cells could be detected in the extracellular environment (Figure 5-4). In summary, the key findings of this in vitro study are that chemotherapy can induce blood cell

apoptosis, and the released caspase-3 in extracellular environment is detectable by caspase-3 assay.

Figure 5-4 Caspase-3 activity assay for cells treated with 1 μ M doxorubicin or 1 μ M paclitaxel for 24, 48, 72 h and the incubation time was 4 h.

A pilot animal study was conducted to examine whether caspase-3 is activated and released into blood stream during the course of chemotherapy regimen and whether this amount of activated caspase-3 in blood can be detected by caspase-3 assay. The result is shown in Figure 5-5.

Figure 5-5 Caspase-3 assay result of plasma sample from mice receiving different cycles of paclitaxel. Mice were given paclitaxel (25 mg/kg) through tail vein injection every 4 days for total 6 cycles. Blood was collected 72 h after drug administration through cardiac puncture.

The plasma samples of cycle 1 and cycle 2 showed red color, because hemolysis occurred during blood collection of cycle 1 and cycle 2. The result shows the caspase-3 activity of samples from cycle 1 and cycle 2 that is indicated by fluorescence intensity of caspase-3 assay is very low. The possible reason is that hemoglobin released into plasma as the result of hemolysis blocked or absorbed the fluorescence emitted by cleaved substrate. In contrast, increase of caspase-3 activity compared to baseline was observed in later cycles of drug administration, especially in cycle 4 and cycle 5. Overall, this result suggests caspase-3 is activated inside cells and released into bloodstream in vivo during chemotherapy process. Moreover, the active caspase-3 present in blood can be detected by caspase-3 assay.

5.2.3. Clinical Study

Thirty-nine women with non-metastatic breast cancer were recruited in this study. Twenty-six women received standard anthracycline (AC)-based regimen (doxorubicin 60 mg/m² in combination with cyclophosphamide 600 mg/m²), given every 21 days for 4 cycles, followed by paclitaxel (Pac) (75 mg/m²), given every 7 days for 12 cycles. Schedule of blood taking was coincided with their routine blood taking – which is: S1 (baseline prior to the start of AC), S2 (prior to 3rd cycle of AC), S3 (prior to the start of Pac), S4 (after 6 cycles of Pac), S5 at the completion of Pac. 13 women received TC regimen (docetaxel 75 mg/m² in combination with cyclophosphamide 600 mg/m²), which was given every 21 days for 4 cycles. Blood collection was scheduled before treatment (S1), after two cycles of treatment (S2) and after whole treatment (S3). The schematic representation of blood collection schedule is shown in Figure 5-6. The endpoints measurement included blood counts (red blood cell, white blood cell and platelet counts), neutropenia episodes requiring

G-CSF support, febrile neutropenia requiring hospitalization, and primary tumor volume.

Figure 5-6 Schematic diagram for blood collection schedule of cancer patients receiving AC-T regimen and TC regimen respectively.

Caspase-3 assay was conducted on samples from each patient. Caspase-3 activity trend within each patient during the whole chemotherapy course was analyzed by comparing active caspase-3 level of subsequent samples with that of baseline sample. Result of caspase-3 assay on plasma samples from the first two patients in AC-T and TC groups respectively is shown in Figure 5-7.

Figure 5-7 Result of caspase-3 assay on clinical samples from the first two patients in AC-T group (TJ/2013/01 and YPG/2013/02) and TC group (LCK/2013/08 and TGL/2013/11) respectively. Caspase-3 activity is normalized to baseline. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ by Student's t test vs. baseline sample.

From this result, elevated plasma caspase-3 activity was observed in cancer patients receiving chemotherapy. Especially in patient YPG/2013/02, increased caspase-3 activity was observed in each plasma sample after drug treatment compared to baseline sample that is collected before commencement of chemotherapy. The highest caspase-3 activity was observed in sample after 4 cycles

of AC treatment that contains anthracyclines with profound myelosuppression effect. This result suggests caspase-3 is activated inside cells and released into blood stream in cancer patients during chemotherapy process. Moreover, the active caspase-3 present in cancer patients' blood can be detected by caspase-3 assay.

Though correlation analysis between caspase-3 activity and absolute neutrophil count (ANC) was confounded by prophylactic G-CSF administration in each patient receiving AC-T regimen to boost white blood cell production, we did observe statistically significant correlation in one subject who received the anthracycline-based regimen (Figure 5-8).

Figure 5-8 Correlation of caspase-3 activity and absolute neutrophil count (ANC) in subject LCH/2013/07 (P=0.03)

In this subject LCH/2013/07, along with the reduction of ANC in cancer patient's blood, the caspase-3 activity was increased continuously during the whole course of AC-T regimen. This result suggests increase of plasma caspase-3 activity may reveal the accumulative hematologic toxicity of chemotherapeutic drugs indicated by decreased ANC in cancer patients.

Statistical analysis of caspase-3 assay results from all 39 cancer patients is shown in Figure 5-9.

Figure 5-9 Statistical analysis of caspase-3 activity in plasma samples from cancer patients receiving chemotherapy.

In summary, clinical samples from 39 breast cancer patients receiving chemotherapy were examined by caspase-3 assay. Significant increase of caspase-3 activity was observed in 21 patients (54%). 26 patients received anthracycline-based (AC-T) regimen, among which 16 patients were observed with significant increase of caspase-3 activity (62%). 13 patients received TC regimen, among which 5 patients were observed with significant increase of caspase-3 activity (38%). In clinical practice, anthracycline-based AC-T regimen induces more serious hematologic toxicity than non-anthracycline ones (TC). Percentage of cancer patients with caspase-3 activity increase in these two therapy groups successfully reflects this clinical situation (62% vs. 38%).

Changes in caspase-3 activity level correlate well with clinically observed drug toxicity (Figure 5-10). In patients receiving anthracycline-based regimen, the average level of caspase-3 activity increased to 125% of baseline level, while the

relative level of WBC dropped to 58% (n=26). In comparison, the average level of caspase-3 activity increased slightly to 106% in patients receiving TC regimen, while the relative level of WBC maintained more stable with lowest level of 87% of baseline value (n=13). Overall, anthracycline-based (AC-T) regimen produced more marked increases in caspase-3 activity level compared with non-anthracycline ones (TC) (125% vs. 106%, $P<0.01$), which is in keeping with the more profound myelosuppression produced by anthracyclines than non-anthracyclines reflected by drops of WBC. (58% vs. 87%, $P<0.001$).

Figure 5-10 Relative levels of caspase-3 activity and WBC in AC-T and TC group. The lowest and the highest value from each patient were used to produce the average value. Highest reading of caspase-3 activity in AC-T group is significantly higher than that in TC group (125% vs. 106%, $P<0.01$), while lowest value of WBC in AC-T group is significantly lower than that in TC group (58% vs. 87%, $P<0.001$). AC-T regimen (n=26); TC regimen (n=13).

Because of severe hematologic toxicity induced by anthracycline-based chemotherapy regimen (AC-T) and insufficient measures to identify patients sensitive to hematotoxicity of anti-cancer drugs, each patient in AC-T group was given with granulocyte colony-stimulating factor (G-CSF) to promote white blood cell production. Even though with this prevention measure, one patient still

developed neutropenia after 4 cycles of anthracycline (AC) treatment with the ANC below 1,500/mm³. Before the occurrence of neutropenia, after 2 cycles of AC treatment, the plasma caspase-3 level was significantly increased to 119% compared to baseline and continuously increased in the subsequent time points (Table 5-2). This result suggests the increase of plasma caspase-3 level may predict the neutropenia occurring in the later stage of treatment course.

Table 5-2 Research data and clinical information of patient LYH/2014/28

LYH/2014/28	AC-1 (Baseline)	AC-3	T-1	T-7	Completion
Normalized caspase-3 activity	100.00	119±6.9*	112±2.1**	119±5.2*	114±3.0**
White cell count (/mm ³)	5800	3300	2300	3300	3400
ANC (/mm ³)	3729	2069	1058	2422	2550
G-CSF administered (Yes or No)	No	Yes	Yes	No	No
Neutropenia	neutropenia				

ANC - absolute neutrophil count
G-CSF - granulocyte colony-stimulating factor

To better reflect the hematologic toxicity of AC-T regimen on cancer patients, the adverse event of patients receiving treatment was defined as both white blood cell count (WBC) and absolute neutrophil count (ANC) were dropped below 50% of baseline value. By this definition, 9 patients out of 26 in AC-T group were observed with adverse event (35%). In these 9 patients with large blood cell count reduction, 4 patients exhibited significant increase of caspase-3 level prior to occurrence of adverse event (44%) (Figure 5-11). This result suggests plasma caspase-3 level may serve as a predictor for hematologic side effect in cancer patients receiving chemotherapy.

Figure 5-11 Plasma caspase-3 level may serve as predictor for hematologic side effect at early stage of treatment.

5.3 Discussion

The clinical research project presented in chapter 5, which is detection of caspase-3 level in blood sample of breast cancer patients during chemotherapy course, is the focus and the most important part of chapter 5. All the conclusions of chapter 5 are made based on data generated from this pilot clinical study. Caspase-3 assay was performed with clinical samples from 39 patients. For each clinical sample, 3 independent experiments were performed to generate data and hence the figures have error bars.

Both the *in vitro* experiment with cells and *in vivo* experiment with mouse model are preliminary studies, the data generated from which are only supporting data for clinical studies. These experiments were only done once and hence the figures have no error bars.

From this study, a few of limitations of the current method were identified. First, Only 25 μ l of plasma can be examined in each run. This limits the sensitivity of caspase-3 assay since caspase-3 is only present in low concentration in the plasma. Second, both the substrate (AC-DEVD-AMC) and the plasma itself have strong

auto-fluorescence and produce high background, which results in low signal-to-noise ratio and further reduce sensitivity of caspase-3 assay on plasma sample. Third, hemolysis, which is hard to avoid completely during blood collection, influence the accuracy of caspase-3 assay. Last, the time point for clinical sample collection is scheduled to align with the routine blood collection of cancer patients, which is at the start of drug administration. At this time point, most of patients have recovered from myelosuppression induced by anti-cancer drugs given in last cycle and hence majority of activated caspase-3 present in plasma may have already been cleared, and caspase-3 activity may have already reduced.

Despite of these above-mentioned limitations, elevated caspase-3 levels in plasma of cancer patients during chemotherapy course were detected by optimized caspase-3 assay. Moreover, the increased level of plasma caspase-3 activity reflected the severity of hematologic toxicity induced by anti-cancer drugs. Cancer patients in anthracycline-based (AC-T) treatment group displayed higher increase in plasma caspase-3 activity compared with non-anthracycline ones (TC), which agrees with the clinical situation that anthracyclines induce more severe myelosuppression than non-anthracyclines reflected by larger reduction of WBC count in patients receiving AC-T. Most importantly, in 4 patients from AC-T treatment group, increase of plasma caspase-3 level was observed prior to occurrence of adverse event that WBC and ANC dropped below 50% of baseline level.

5.4 Conclusion

In summary, through this pilot clinical study, caspase-3 level in plasma was shown to potentially provide a measurement of hematologic toxicity of

chemotherapy and to even potentially serve as a predictor for hematologic adverse event occurred in individual cancer patient receiving anthracycline-containing chemotherapy regimen. Therefore, caspase-3 level in plasma may have potential to guide dosing schedules of anti-cancer drugs as well as preventive measures for hematologic adverse event in clinical practice.

Chapter 6 Conclusions and Future

Directions

This thesis focuses on both effectiveness and safety of anthracycline administration for breast cancer treatment, which greatly determines the treatment outcome for TNBC patients.

In the first part (chapter 3 and chapter 4), 2D-DIGE proteomics was applied to compare the protein profiles between a TNBC cell line MDA-MB-231-C3 (231-C3) that expresses apoptosis sensor and its lung metastatic cell line 231-M1 that displayed significantly higher cell viability and lower apoptotic rate than 231-C3 cells upon DOX treatment *in vitro*. G6PD, an important antioxidant protein present in cytoplasm, was demonstrated to up regulate in 231-M1 cells compared with 231-C3 cells. Interfering with G6PD expression by siRNA or inhibiting its activity with its chemical inhibitor DHEA successfully reduced cell viability and increased apoptotic rate of the metastatic cell line 231-M1 as well as multiple original TNBC cell lines. Clinical database analysis revealed that TNBC patients with relatively higher G6PD expression exhibited lower overall survival rate than lower G6PD expression patients, which supports the hypothesis that high G6PD expression confers breast cancer cells with survival advantage on chemotherapy treatment and hence results in low survival probability of cancer patients. Therefore, it was concluded that G6PD mediates metastasis acquired and intrinsic DOX resistance of TNBC cells. Moreover, G6PD is a potential therapeutic target for sensitizing TNBC towards DOX treatment.

G6PD has been suggested to be the prognostic markers of TNBC. High expression of G6PD is associated with worse overall survival and progression-free survival in TNBC [169]. In another research work, the DOX-resistant human colon cancer cells that showed increased activity of pentose phosphate pathway (PPP) and glucose-6-phosphate dehydrogenase (G6PD) have lower intracellular DOX accumulation in comparison with DOX-sensitive counterpart. The author concluded that the high intracellular glutathione (GSH) level generated by activated PPP and increased G6PD activity helps cells to extrude DOX out of cells [57].

Based on these previous studies, the study in this thesis demonstrated for the first time that suppression of G6PD abrogates DOX resistance of metastatic TNBC cells, and also increases sensitivity of multiple TNBC cell lines to DOX treatment. Furthermore, this study explored the underlying mechanism of G6PD-rendered DOX resistance of TNBC cells. Distinct from previously published study, high G6PD expression does not affect intracellular DOX concentration but protect cancer cells from oxidative stress generated by DOX treatment and hence promote cancer cell survival.

DHEA is a well-known uncompetitive G6PD inhibitor, which exerts its inhibition effect by binding to G6PD ternary enzyme-substrate complex [151]. It has been commonly used as G6PD inhibitor in various research works, including some high impact research works [118, 153]. However, the clinical application of DHEA as G6PD inhibitor is extremely limited by its high hydrophobicity. As DHEA is sparingly soluble in aqueous buffers, its administration by i.v. injection is quite challenging. Through oral administration, the plasma concentration of DHEA is extremely low. It was reported that after 6 months of daily DHEA (200 mg/day) oral administration to healthy young men, the maximal plasma concentration is only

69 nM, which is dramatically lower than 200 μ M concentration used to treat TNBC cells in my study [170].

According to literature analysis, a few of research works have been focusing on development of novel and potent G6PD inhibitors. Some promising compounds have been found to be 100- to 1000-fold more effective than DHEA on G6PD inhibition with different mechanisms [164, 171, 172].

Following these published works, these novel G6PD inhibitors could be tested *in vitro* study of treating TNBC cells with DOX. The ones showing most potent synergistic effect with DOX could be assessed with *in vivo* study to determine their toxicity and efficacy before initiation of clinical trials on human. It is expected that the G6PD inhibitors can be administrated by intravenous infusion before DOX is given to cancer patients for suppressing G6PD activity in cancer cells in advance of DOX treatment. In this way, the DOX resistance of cancer cells would be reduced and the efficacy of DOX would be maximized.

The second part (chapter 5) of this thesis is to develop the caspase-3 activation as an assessment means and early indicator for hematologic toxicity in breast cancer patients receiving chemotherapy containing anthracyclines. 39 female non-metastatic breast cancer patients were recruited in this project. Elevated plasma caspase-3 levels in cancer patients during chemotherapy course were successfully detected by optimized caspase-3 assay. Moreover, the increased level of plasma caspase-3 activity reflected the severity of hematologic toxicity induced by anti-cancer drugs. Cancer patients in anthracycline-based (AC-T) treatment group displayed higher increase of plasma caspase-3 activity compared with non-anthracycline ones (TC), which agrees with the clinical situation that anthracyclines induce more severe myelosuppression than non-anthracyclines reflected by larger

reduction of white blood cell (WBC) count in patients receiving AC-T. Most importantly, in 4 patients from AC-T treatment group, increase of plasma caspase-3 level was observed prior to occurrence of adverse event that defined as WBC count and absolute neutrophil count (ANC) dropped below 50% of baseline level. In conclusion, through this clinical study, plasma caspase-3 level in cancer patients under chemotherapy treatment is suggested to be a potential indicator of hematologic toxicity induced by anthracyclines-containing chemotherapy for the first time. Moreover, caspase-3 level in plasma is suggested to potentially serve as a predictor for hematologic adverse event in cancer patients and hence shows potentiality in guiding dosing schedules of anthracycline drugs and preventive measures for hematologic adverse event in individual cancer patient.

In future work, new method that can detect caspase-3 level inside plasma with high sensitivity and efficiency needs to be developed. Caspase-3 enzyme from patient's plasma can be isolated and harvested using immunoprecipitation in order to increase the concentration of caspase-3 and to reduce background fluorescence.

Animal study should be conducted with larger sample size. Anti-cancer drugs that can induce more severe hematologic toxicity, such as doxorubicin, should be administered into mice with more cycles.

Correlation study between caspase-3 level and adverse event occurred in cancer patients needs to be conducted based on optimized blood collection time point. In an ideal condition, plasma caspase-3 levels at different time points post anti-cancer drug administration need to be compared to obtain an optimal blood collection schedule. Blood collection of all cancer patients recruited in clinical study should comply with this optimized schedule.

Clinical study should be conducted with larger cancer patient size. Patients with different kinds of cancers such as breast and colorectal cancers, and treated with different anti-cancer drugs including anthracyclines, taxanes, platins and 5-fluorouracil could be recruited. The clinical hematologic adverse event, such as severe neutropenia requiring dose reduction and febrile neutropenia requiring hospitalization should be correlated with plasma caspase-3 level in cancer patients to validate the indication and prediction value of caspase-3 level for hematologic toxicity of chemotherapy in cancer patients.

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