Multidrug Resistance and Breast Cancer

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1. Introduction

Millions of new cancer patients are diagnosed each year and over half of these patients die from this disease. As the second leading cause of cancer deaths, breast cancer is estimated to be diagnosed in over one million people worldwide and to cause more than 400,000 deaths each year [1]. Chemotherapy is part of a successful treatment to many cases; however, the development of multidrug resistance (MDR) to it becomes a major obstacle so as to as few as half of the breast cancer patients treated benefit from chemotherapy.

MDR is a term used to describe the phenomenon characterized by the ability of drug resistant tumors to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents [2]. The cytotoxic drugs that are most frequently associated with MDR are hydrophobic, amphipathic natural products, such as the taxanes (paclitaxel and docetaxel), vinca alkaloids (vinorelbine, vincristine, and vinblastine), anthracyclines (doxorubicin, daunorubicin, and epirubicin), epipodophyllotoxins (etoposide and teniposide), antimetabolites (methotrexate, fluorouracil, cytosar, 5-azacytosine, 6-mercaptopurine, and gemcitabine) topotecan, dactinomycin, mitomycin C and so on [3].

At present, many mechanisms have been found to be responsible for it, including overexpression of the members of the adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter family, changes of apoptosis-related genes, the alteration of DNA-repair gene, cancer stem cells and so on. And up to date, many methods were adopted to overcome MDR, for example natrual drugs, chemical drugs and genetic therapy. Herein, we will introduce the mechanisms and therapy of MDR of breast cancer briefly.

2. Mechanisms of MDR

2.1 The adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter family

Elevated expression of ATP-binding cassette (ABC) transporters is considered to be the main cause of MDR in breast cancer. ATP-binding cassette (ABC) transporters are a family of transporter proteins that contribute to drug resistance via adenosinetriphosphate (ATP)-dependent drug efflux pumps, which can result in an increased efflux of the cytotoxic drugs from the cancer cells, thus lowering their intracellular concentrations [4]. Up to date, more than 100 ABC transporters from prokaryotes to humans and 48 human ABC genes have been identified that share sequence and structural homology [3]. The proteins which are related to the MDR in breast cancer are mainly including p-glycoprotein (p-gp), multidrug resistance-related protein (MRP) and breast cancer resistance protein (BCRP).
In mammals, the functionally active typical ABC proteins consist of at least four core domains, two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The two TMDs of each ABC transporter consist of multiple membrane-spanning \( \alpha \)-helices (typically, but not always, six \( \alpha \)-helices per domain) and form the pathway through which substrate crosses the membrane. The two NBDs play a role in cleaving ATP (hydrolysis) to derive energy necessary for transporting cell nutrients such as sugars, amino acids, ions and small peptides [5]. Normally, they have important physiological function, such as the excretion of toxins from the liver, kidneys, and gastrointestinal tract [6]. Overexpression of these transporters has been observed in many types of human malignancies and correlated with poor responses to chemotherapeutic agents.

### 2.1.1 P-glycoprotein

In 1970s, a carbohydrate-containing protein, 170 kDa in molecular weight, was found in multidrug-resistant Chinese hamster ovary cells. The glycoprotein was named P-glycoprotein (P-gp) because the protein can modulate membrane permeability with respect to a number of apparently unrelated drugs including actinomycin D, methotrexate, daunomycin, and colchicine [7]. The MDR mediated by P-gp is also called” classical MDR”. Gene sequence membrane analysis for mammalian P-gp has revealed the presence of two similar halves, each containing 6 putative transmembrane segments, and an ATP-binding consensus motif. The human protein is comprised of 1280 amino acids with 12 transmembrane domains and 43% sequence homology between the two halves. Three glycosylation sites on the first extracytoplasmic domain are present [8]. The gene encoding p-glycoprotein was termed mdr1. The gene with 28 exon and 1.2 kb and is located on chromosome 7q21.12.

There are three known isofoms of P-gp, namely, class I, II and III. Rodent cells have all three P-gp genes, whereas human cells only have class I and III P-gp [9]. Classes I and II P-gp genes confer MDR when transfected into sensitive wild type (WT) cells, whereas the class III P-gp gene is not shown to be associated with drug resistance. All three types of P-gp expressed in several normal tissues. In mammalian tissues, class I P-gp is found in epithelium, intestinal, endothelial cells, bone marrow progenitor peripheral blood lymphocytes, natural killer cells and so on. The class III P-GP is localized in hepatocytes, cardiac and striated muscle [10]. The distribution displays that p-gp plays an important role in normal physiological function. Evident confirmed that P-gp take part in the transepithelial secretion of substrates into bile, urine, or gastrointestinal tract lumen. P-gp may also confer a protective role to mediate xenobiotic efflux in tissues such as the brain, testis, and placenta.

P-gp substrates are widespread. Although their structures are very different, they share many physical properties including high hydrophobicity, an amphiphilic nature and a net positive charge[11]. It is important for us to understanding the modulation of P-gp. Evident shows that p-gp is phosphorylated by protein kinase C (PKC) and PKC blockers can reduce P-gp phosphorylation and increase drug accumulation. However, there is evidence that PKC inhibitors directly interact with P-gp and inhibit drug transport by a mechanism independent of P-gp phosphorylation [12]. Experiments using transient transfection of the MDR1 promoter region (linked to a reporter gene) into the cells as well as stable transfection of some other genes showed that genes p53, ras and raf can influence the activity of introduced MDR1 promoter or the expression of the endogenous cellular MDR1. Genes c-fos and c-jun also were shown to confer the regulation of MDR1 activity [13].
For a long time, P-gp was believed to be the only protein capable of conferring MDR in mammalian tumor cells. Over 50% breast cancer expressed P-gp [14]. Moreover, prior exposure to chemotherapy or hormonal therapy has been shown to increase the proportion of breast cancers expressing P-gp by 1.8-fold [15]. However, pre-chemotherapy P-gp expression showed no association with shorter progression-free survival (PFS) so the clinical relevance of this observation in terms of screening patients and treatment selection remains unclear[16].

2.1.2 Multidrug resistance-related protein 1 (MRP)
In 1992, Susan Cole and Roger Deeley observed amplification and increased expression of a novel gene in non-P-gp expressing small cell lung cancer DOX resistant cell lines and this is the MRP1 (ABCC1) (MDR related protein) gene. The following study shows that the protein encoded by this gene is also a member of ABC transporters [17].

The multidrug-resistance-associated protein (MRP or MRPI) is a 190 kDa protein and is constituted by 1531 amino acids. Like other members of ABC transporters, MRPI has 3 membrane spanning domains, 2 NBDs and extracellular N-terminal. Up to now, several isoforms of MRPI have been identified. Included among these are five human MRPI-related proteins, designated MRP2, MRP3, MRP4, MRP5 and MRP6. MRP7, MRP8 and MRP9 are recent additions to the family which have not yet been characterised[18].

Physiologically, MRPI also plays a normal role in the ATP-dependent unidirectional membrane transport of glutathione conjugates, such as leukotriene C4, S-(2,4-dinitrophenyl)glutathione and leukotriene receptor antagonists could inhibit this function[19]. Besides multidrug-resistance cancer cells, MRPI is also expressed in normal human tissues, such as muscle, lung, spleen, bladder, adrenal gland and gall bladder [2]. MRP2 (or cannalicular multispecific organic anion transporter or cMOAT) was first shown to be expressed in the liver which functions in the excretion of glutathione and glucuronate conjugates across the cananlicular membrane into bile. In addition, MRP2/cMOAT is also expressed in the human kidney proximal tubule epithelia on the apical side. Therefore, it is implicated that MRP2 may play a role in the renal excretion of endogenous substances and xenobiotics, in normal conditions. MRP3 is expressed in liver and involved in the efflux of organic anions from the liver into the blood in case of biliary obstruction. MRP4 and MRP5 transport nucleosides and confer resistance to antiretroviral nucleoside analogs. MRP6 is a lipophilic anion pump with a wide spectrum of drug resistance. Among the members of MRP family, only MRPI has been widely accepted to cause clinical drug resistance[3].

Like other members of ABC transporters, MRPI can pump anti-tumor drugs out of the tumor cells, cause reduced intracellular accumulation of drugs and lead to resistance. Whereas P-gp transports neutral and positively charged molecules in their unmodified form, MRPI overexpression is associated with an increased ATP-dependent glutathione-S conjugate transport activity. Reduced glutathione (GSH) has been suggested as an important component of MRPI mediated MDR and drug transport. MRPI is able to transport a range of substrates as such or conjugated to GSH, glucuronide, and sulfate [20]. The anticancer drugs that are substrates of MRPI mainly include anthracyclines such as doxorubicin and daunorubicin, vinca alkaloids and etoposide. Several findings indicate that MRPI reduces drug accumulation by effluxing drugs by a GSH co-transport mechanism or after their conjugation to GSH [21]. But the mechanism by which GSH facilitates transport of some compounds by MRPI is still a matter of debate.
2.1.3 Breast cancer resistance protein (BCRP)

Breast cancer resistance protein (BCRP) is the latest ABC transporter involved in MDR and it was cloned by Ross and Doyle in 1998 from a mitoxantrone-resistant subline of the breast cancer cell line MCF-7/Adr/Vp which does not express other known multidrug efflux transporters such as P-glycoprotein (P-gp) or the multidrug resistance protein 1 (MRP1) [22]. Two almost identical proteins as BCRP with only a few amino acid differences were later discovered independently by other laboratories from mitoxantrone-resistant human cancer cell lines (so named as MXR) and human placenta (so named as ABCP)[23].

The BCRP gene is located on chromosome 4q22. The full length of BCRP gene is 66kb and the length of mRNA is about 2.4kb [23]. The product of the gene is a 72KD protein with 655 amino acid that contains an ATP-binding domain and six transmembrane domains, and it is a half transporter member of the ABCG subfamily [24]. As a half transporter, BCRP functions as a homodimeric/oligomeric efflux pump [25], and in a manner that is similar to other ABC transporters. Besides that, BCRP can also transport hydrophilic conjugated organic anions, particularly the sulfated conjugates with high affinity, for example BCRP can detoxify irinotecan and SN-38 by glucuronidation via the activity of UDP-glucurononyltransferase [26]. BCRP substrates include not only chemotherapeutic agents such as mitoxantrone, methotrexate, topotecan, irinotecan and its active analog SN-38, and tyrosine kinase inhibitors imatinib and gefitinib, but non-chemotherapy drugs such as prazosin, glyburide, nitrofurantoin, dipyridamole, statins, and cimetidine as well as nontherapeutic compounds such as the dietary flavonoids, porphyrins, estrone 3-sulfate (E1S), and the carcinogen PhIP [27].

Similar to P-gp and MRP1, BCRP is widely expressed in normal cells and tissues including the capillary endothelial cells, the hematopoietic stem cells [28], the maternal–fetal barrier of the placenta and the blood-brain barrier [29]. In these tissues, BCRP play a protective role against xenobiotics and their metabolites. Whereas, the apical localization of BCRP in the intestinal epithelium and in the bile canalicular membrane also suggests the intestinal absorption and hepatobiliary excretion of BCRP substrates [30].

Unexpectedly, many mutant forms of BCRP proteins were found in drug-selected cells such as those of the S1-M1-80 and MCF7/AdVp3000 cell lines and up to now, more than 50 mutations have been identified including natural variants and non-natrual mutations [27]. The most important natural variant is Q141K, which occurs in Japanese and Chinese populations at high allele frequencies (30–60%) and in Caucasians and African-American populations at relatively low allele frequencies (5–10%) [27]. Several studies consistently revealed that Q141K had a lower protein expression level than wild-type BCRP in both transfected cells and human tissues. A recent study has revealed that Q141K undergoes increased lysosomal and proteasomal degradations than wild-type BCRP, possibly explaining the lower level of protein expression of the variant [31]. The R482T and R482G variants of BCRP detected from MCF7/AdVp3000 and S1-M1-80 cells belong to non-natural mutants. The non-natural mutants have different effects on BCRP expression, distribution and functions. Some mutations do not affect plasma membrane expression, but alter substrate specificity and/or overall transport activity. For example, the R482T and R482G lose their methotrexate-transporting activity but at the same time confer increased mitoxantrone resistance, so they are highly resistant to both mitoxantrone and doxorubicin[32]. Wild-type BCRP does not transport Rhodamine 123 and Lyso-Tracker Green; however, the mutants R482T and R482G do [33]. These findings confirmed that the
transmembrane region of BCRP plays important roles in its activity. Some mutations affect biogenesis with decreased stability, lower expression and/or altered subcellular distribution of BCRP. A typical example is mutations of Arg383 which results in a significant decrease in the protein level, partial retention in the endoplasm reticulum, and altered glycosylation and the treatment with mitoxantrone assisted in protein maturation [34]. Some mutations influence the chemical modifications of BCRP such as N-linked glycosylation or disulfide bond formation in BCRP such as the mutation Asn596. Otherwise, there are also many mutations which do not have major effects on both plasma membrane expression and function of BCRP, including K473A and H630X. The research on the mutations of BCRP could help us to further understand the structures and functions of ABC transporters.

Up to date, BCRP was detected in many resistance tumor cells such as human colon cancer cell line S1-M1-80, prostate cancer cell lines and breast cancer cell line MCF7/AdVp3000 [35]. Many clinical sample were also found BCRP expression, including acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), non-small cell lung cancer and so on[36-38]. And it has been suggested that the expression of BCRP is associated with a poor response to cancer chemotherapy and may be responsible for clinical drug resistance. However, the studies on the expression characters of BCRP in breast cancer clinical samples are still very few.

2.2 Apoptosis and MDR
2.2.1 P53
As a tumor suppressor, p53 plays a pivotal role in inducing apoptosis in response to cellular damage, including DNA damage. However, mutant P53 plays an opposite role in the regulation of apoptosis, that is mutant p53 is an anti-apoptosis factor. In a study from the National Cancer Institute (NCI), the majority of breast cancer cell lines were mutant for p53 [39]. About 50% of all tumours have an approximately 25% occurrence of deletions and point mutations in sporadic breast cancers[40]. Many anti-tumor drugs can lead cellular death by inducing cellular apoptosis. When p53 mutations or deletions occur, the cellular apoptosis can be inhibited and the cells exhibit MDR phenotype. Mutations in p53 have been verified to be related with resistance to doxorubicin in breast cancer patients[41].

Many data show the correlation of P53 and ABC transporters. The first experiments implied that a mutant p53 (mtP53)specifically stimulated the MDR1 promoter and wild-type p53(wtP53) exerted specific repression[42]. In the follow-up study, a p53 consensus binding sequence was also found in the promoter of the rat ABCB1 gene. Both promoter function and endogenous mdr1b expression were shown to be up-regulated by wtP53[43]. More studies displayed that the mutations of p53 can dramatically activate the ABCB1 promoter in multiple cell lines including Saos-2, Caco-2, MCF-7 and so on[44]. Linn et al. assessed the status of p53 and ABCB1 in both primary operable and advanced-staged tumors and their results revealed that nuclear p53 accumulation and coexpression of ABCB1 were more prevalent in locally advanced breast cancers and that these markers provided a strong prognostic indication of shorter survival[45, 46]. Similar results were also found in other studies.

Recently, Wang et al investigated the effects of wild-type and mutant p53, and nuclear factor kappa-B (NF-kappaB) (p50) on BCRP promoter activity in MCF-7 cells, and the results show that wild-type p53 induced transcriptional suppression of breast cancer resistance protein (BCRP) through the NF-kappaB pathway in MCF-7 cells[47].
2.2.2 Other apoptosis related genes and MDR
Mitochondrial (intrinsic pathway) and cell surface receptor (Fas) mediated (extrinsic pathway) apoptosis are the two main routes leading to programmed cell death. MCF-7 cells can undergo apoptosis by the sequential activation of caspases-9 (associated with mitochondrial mediated apoptosis), -7, and -6. Recently, a splice variant form of caspase-3 has been shown to be overexpressed in chemoresistant, locally advanced breast cancers, and is particularly associated with response to cyclophosphamide[48].

Bcl-2 is a member of a large family of genes coding both anti-apoptotic proteins (for example, Bcl-2, Bcl-XL) and pro-apoptotic proteins (Bax, Bad, Bic, etc.). Bcl-2 protein is able to inhibit the apoptosis induced by p53 in response to genotoxic stress. There are data showing that Bcl-2 overexpression results in the resistance of cells to different drugs, including DOX, taxol, etoposide, camptothecin, mitoxantrone and cisplatin[49]. When Bcl-2 is over expressed and contributes as a resistance mechanism, it has been shown that the anticancer drugs promote cell cycle arrest; however, their effects are cytostatic rather than cytotoxic[2]. The phosphorylation state of the Bcl-2 oncoproteins has been shown to modulate response to taxanes[50].

Survivin is another apoptosis-related gene which has been confirmed to confer MDR in tumors. It is a structurally unique inhibitor of apoptosis (IAP), substances which block apoptosis induced by a variety of nonrelated apoptosis triggers. Survivin is known to directly or indirectly bind and inhibit the terminal effector cell death protease cascades, caspase 3 and 7, as well as inhibit the activation of caspase 9[51]. Furthermore, it has been reported that the expression of survivin was significantly higher after treatment with anticancer drugs in many cancer cells and may be involved in radio- and chemo-resistance[52]. Liu et al. documented that survivin might modulate the turnover of P-gp or transport by P-gp in the cell, which then resulted in anti-apoptosis and drug resistance in breast cancer cells[51]. However, the role of survivin in MDR breast cancer in the presence of P-gp is still not clear.

In addition, some other apoptosis-related genes were found to take part in the regulation of MDR, such as CD95, TRAIL and so on.

2.3 MDR-related enzyme
2.3.1 Glutathione S-transferase (GST)
GST is a member of phase II detoxification enzymes that catalyses the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds. Because of their capacity to react with electrophiles, radicals and reactive oxygen species, GSTs, together with GSH, have a major role in the protection against oxidative stress [53].

GSTs are divided into two super-family members: the membrane microsomal and cytosolic GSTs (c-GSTs). Microsomal GSTs (m-GSTs) are structurally distinct from the cytosolic in that they homo- and heterotrimerize rather than dimerize to form a single active site and the microsomal GSTs are mainly involved in the metabolism of endogenous compounds, like leukotrienes and prostaglandins. The cytosolic GSTs also conjugate exogenous compounds and the cytosolic GSTs are subject to significant genetic polymorphisms in human populations. Up to date, the cytosolic GSTs are divided into seven classes, Alpha (A), Mu (M), Omega (O), Pi (P), Sigma (S), Teta (T) and Zeta (Z) which have a promiscuous substrate specificity and are localized in different tissues with organ specific expression patterns [54]. The GST-Pi have been confirmed to be closely related with MDR.
Many data show that GST confers the development and expression of MDR. Increased expression of GSTπ—detected as strong immunoreactivity—has been documented to contribute to drug resistance of ovarian carcinomas, head and neck cancer, lung squamous-cell carcinoma, breast cancers and so on[55]. Cells with GST isozyme transfections yield mild increases in resistance (mostly in the 2–5 fold range) to a number of different anticancer drugs [54]. While inhibition of GST expression by antisense cDNA increased the sensitivity to several anticancer drugs [56]. Besides, exposure of cells to a specific inhibitor of c-GCS, buthionine sulfoximine (BSO), decreases multidrug resistance to doxorubicin and vincristine[57]. The substrates of GST reported include chlorambucil, melphalan, nitrogen mustard, phosphoramide mustard, acrolein, carmustine, hydroxyalkenals, ethacrynic acid and steroids. And the MDR mediated by GST is related to mitomycin C, adriamycin, cisplatin and carboplatin.

How GSTs affect MDR in tumor cells? There are mainly two mechanisms found now. First, GST-Pi plays a key role in regulating the MAP kinase pathway via protein: protein interactions. GST-Pi was shown to be an endogenous inhibitor of c-Jun N-terminal kinase 1 (JNK1), a key member of MAP pathway which involved in stress response, apoptosis, and cellular proliferation [58]. In nonstressed cells, low JNK activity is observed due to the sequestration of the protein in a GST-Pi : JNK complex. Direct protein : protein interactions between the C-terminal of JNK and GST-pi were reported with a binding constant of approximately 200 nm. The second, there is a coordinate action of phase II enzymes and MRP in MDR[59]. The already mentioned connection between a MRP drug resistance profile and an increased GST-pi expression, shown in many cell lines, is indeed indicative for a shared regulatory mechanism of MRP and GSTs expression [60]. Studies demonstrated that Nrf2 may be play a key role between MRP and GSTs. A study on Nrf2 knockout mice displayed that: disruption of the Nrf2 gene decreased both the constitutive as well as the inducible expression of class Alpha, class Mu and class Pi glutathione transferases[61]. Meanwhile, Nrf2 was also shown to be necessary for the constitutive and inducible expression of MRP1 in mouse embryo fibroblast[62].

GSTπ immunoreactivity was reported not to correlate with response to chemotherapy in cervical carcinoma, but many data show that in primary breast cancers, expression of GST-Pi was associated with poor prognosis. Fengxi Su et al analyzed the relationship between GST-Pi and the FAM (5-fluorouracil, adriamycin, mitomycin) protocol and the result showed that the presence of GSTπ in breast cancer tissue was a bad prognostic indicator, and these tumors were largely resistant to chemotherapy[55]. In cultured breast cancer cells, GST-pi is exclusively expressed in estrogen receptor-negative (ER−) cells but not in receptor-positive (ER+) cells [63]. In 1997, Mona S. Jhaveri verified that that methylation status of the promoter contributes significantly to the levels of GSTP1 expressed in ER− and ER+ breast cancer cell lines [64].

2.3.2 DNA topoisomerase II (topo II)

DNA topoisomerase II (topo II) is a nuclear phosphoprotein involved in DNA replication and chromosome dynamics. These enzymes catalyse the ATP-dependent passage of one DNA duplex (the transport or T-segment) through a transient, double-stranded break in another (the gate or G-segment), navigating DNA through the protein using a set of dissociable internal interfaces, or 'gates' [65,66]. The family of DNA topoisomerase II includes two related but genetically distinct isoforms isforms TOPIIα and IIβ in mammalian cells.
The human topoisomerase IIα gene (TOP2α) is localized on chromosome 17q21-22 [67] whereas TOP2β maps to chromosome 3p24 [68]. The cDNAs for the human α and β isoforms encode p170 and p180 proteins of 1531- and 1621-amino-acid [68], respectively. TOP2α lies close to the epidermal growth factor-like receptor gene ERBB2 (HER2) and the retinoic acid receptor locus RARK in a region of chromosomal 17 which is amplified in some human breast cancer [69]. The two enzymes are closely similar in structure each comprising three functional domains defined by sites of cleavage by trypsin or staphylococcal V8 proteases: an N-terminal ATPase domain (approximately residues 1-400); a DNA breakage-reunion region (400-1220); and the C-terminal domain which carries a multitude of phosphorylation sites[70].

In addition to its role in cell division, TOP2α is also found to be related to the MDR in tumor. It is the major molecular target for a large group of clinically relevant, structurally different cytotoxic agents known as TOP2α inhibitors including the anthracycline class of antitumor cytotoxic agents [71]. These drugs all act by forming covalent bonds with TOP2α, creating a complex that introduces permanent double-strand breaks in DNA leading to apoptosis. Reduced topoisomerase II expression or function can contribute to resistance to agents such as anthracyclines and epipodophyllotoxins [72]. In vitro studies of breast tumor cell lines have shown that amplification of the TOP2α gene leads to protein overexpression and sensitivity to anthracyclines [73,74]. Similarly, deletion of TOP2α genomic alterations in breast cancer leads to a marked decrease in TOP2α protein expression, which results in chemoresistance to TOP2α inhibitor anticancer drugs in cell culture.

The HER-2 gene is another gene on chromosome 17 and it encodes for a ligandless, transmembrane glycoprotein receptor with intrinsic tyrosine–kinase activity. HER-2 gene amplification or protein overexpression occurs in about 20% of patients with breast cancer and is a recognized poor prognostic marker, often associated with endocrine resistant, high grade disease[75]. Recently research reported that the expression of TOP2α is closely related to the expression of HER2 gene and co-expression of them may be a useful tool in predicting benefit from chemotherapy. Top IIα is reported to be either amplified or deleted in nearly 90% of HER-2 amplified primary breast cancers[76]. Recent review of the Canadian-MA.5 trial assessed TOP2α alterations and HER-2 amplification by FISH on tissue microarrays in 438 patients [77]. Top IIα alterations occurred in 18% patients (12% amplification, 6% deletions) and were more common in large tumors and in HER-2 positive tumors. In patients with Top IIα alterations, relative benefits of therapy were seen with CEF having statistical superiority over CMF in terms of RFS (adjusted HR 0.35, 95% CI 0.17 - 0.73, p = 0.005) and OS (adjusted HR 0.33, 95% CI 0.15 - 0.75, p = 0.008). However, there are also diffusing evidents. An analysis displayed topo IIa mRNA overexpression in 19% of HER-2 negative patients [78]. In conclusion, the relationship between TOP II, HER2 and chemosensitivity needs further investigation.

2.3.3 Glucosylceramide synthase
Sphingolipids, which include ceramides and sphingosine, were first isolated and characterized in the late 1800s. Recent years, many studies have shown that they are not only structural and insert components of cell membranes but also associated with myriad process of cells including the proliferation, survival and death of cells. As an important member of sphingolipid metabolism, ceramide have been proven to be a second messenger of apoptosis [79, 80]. Cellular stress is known to increase ceramide levels in cells. So it is easily
to understand that increased ceramide has been observed in response to many anti-cancer drugs, such as doxorubicin, vincristine, paclitaxel, etoposide, PSC 833 and fenretinide. Many enzymes have been confirmed to be responsible for the regulation of ceramide levels, such as ceramide synthase and sphingomyelinase which are responsible for the ceramide generation, and sphingomyelin synthase and ceramidase which take part in the ceramide metabolism[81]. Glucosylceramide synthase (GCS) is one of them. As an enzyme which catalyzes the first step in glycosphingolipid synthesis, GCS transfers UDP-glucose to ceramide to form glucosylceramide, which have been found to involve in many cellular processes such as cell proliferation, oncogenic transformation, differentiation, and tumor metastasis[82]. In addition, many studies show that GC was related with MDR in many tumor cells. In 1996, Lavie Y et al first reported that chemotherapy resistant MCF-7-AdrR breast cancer cells accumulate GC in comparison to wild-type MCF-7 cells[83]. After that, GC was found to confer to MDR in many other cancers [84-86]. So some people guessed that elevated GCS activity may be a novel form of multidrug resistance.

Then, Liu etc found that increased competence to glycosylate ceramide conferred adriamycin resistance in MCF-7 breast cancer cells by transfection with GCS cDNA[8], while using GCS inhibitor 1-phenyl-2-palmitoylamino-3- morpholino-propanol (PPMP) or transfection of doxorubicin-resistant MCF-7-AdrR cells with GCS antisense both restored cell sensitivity to doxorubicin or vinblastine and paclitaxel[86,87]. Ladisch found that blocking GCS with D, L-threo-phenyl-2-hexadecanoylamino-3-pyrrolidino-1- propanol (PPPP), was able to elevate ceramide levels and enhance vincristine cytotoxicity via programmed cell death[88]. All the following works demonstrated that GCS was potentially one MDR-related drug resistance mechanism.

Recently, Yong-Yu Liu et al reported that glucosylceramide synthase upregulates MDR1 expression in the regulation of cancer drug resistance through cSrc and β-catenin signaling in the ovarian cell line NCI/ADR-RES which was ever named MCF-7/AdrR [89]. This study revealed the importance of GCS in the mechanism of cancer drug resistance.

Further studies demonstrated that a GC-rich/Sp1 promoter binding region was of importance in the regulation of GCS expression and doxorubicin could induce activation of Sp1 and up-regulation of GCS and apoptosis in Leukimia drug-resistance cell line HL-60/ADR and ovarian cell line NCI/ADR-RES [81,90].

In 2009, Eugen Ruckhäberle et al analyzed microarray data of GCS expression in 1,681 breast tumors and found that expression of GCS was associated with a positive estrogen receptor (ER) status, lower histological grading, low Ki67 levels and ErbB2 negativity (P < 0.001 for all) [91]. This study revealed the expression profile of GCS in breast cancer. But, the study also found that GCS has no clearly correlation with mdr1. So the relationship between GCS and mdr1 in breast cancer is still a puzzle.

2.4 Cancer stem cells and MDR

Stem cells are defined as cells with both self-renewal capacity and the ability to produce multiple distinct differentiated cell types to form all the cell types that are found in the mature tissue[92]. Thus, these two characteristics of stem cells confer the unique property of asymmetric division. Stem cells are quiescent or slowly cycling cells maintained in an undifferentiated state until normal functioning of the organism needs their participation. Stem cells are classified into two principal types: embryonic and adult stem cells[93].
Recent studies have revealed that they play important role in cancer biology. Cancer stem cells (CSC) have been detected in many tumors, such as retinoblastoma and melanoma [94,95]. In breast cancer, a CD44+/CD24-or low/Lin- cell population was first identified as CSC [96]. Later, aldehyde dehydrogenase (ALDH) 1 activity was reported to be associated with stem/progenitor properties in breast cancer [97]. Although the origin of cancer stem cells has not yet been elucidated, researchers proposed that the malignant transformation of a normal stem cell, or a progenitor cell that has acquired self-renewal ability may be the reason. Up to now, Three major pathways have been identified to be related with the regulation and maintenance of stem cells in adult life: Wnt, Hedgehog, and Notch[92].

It is well known that cancer chemotherapy targets dividing cells. Because stem cells are quiescent or slowly cycling cells under normal situation, it is easy to understand that cancer stem cells could escape from the killing of anti-tumor drugs. Besides, the side-population (SP) cells may be another reasons why stem cells become multi-drug resistant. The isolation of SP cells is based on the technique described by Goodell et al. in 1996 [98]. While experimenting with staining of murine bone marrow cells with the vital dye, Hoechst 33342, they discovered that the display of Hoechst fluorescence simultaneously at two emission wavelengths (red 675 nm and blue 450 nm) localizes a distinct, small, nonstained cell population (0.1% of all cells) that express stem cells markers (Sca1+linneg/low), which were named SP cells. At first, they thought the exclusion of Hoechst 33342 by SP cells is an active process involving multidrug resistance transporter 1 (MDR1). But the following study show that MDR1 can not be taken as a single marker to identify and isolate SP cells. Zhou et al. have demonstrated the breast cancer resistance protein (BCRP) may also attend the SP phenotype[28]. The SPs from breast cancer contain primitive stem cell-like cells that can differentiate into epithelial tumors in vitro and in vivo and express stemness genes [28,99]. The characterization of cells within SP demonstrates that they are immature, poorly differentiated, and highly tumorigenic. Gene expression profiles of SP show that these cells are less differentiated than non-SP cells [100]. The ABC transporters may play three functions in CSCs. First, the ABC transporters can protect the CSCs against exogenous products able to penetrate the cell membrane barrier by active exclusion. Second, there is mounting speculation that ABC transporters repress the maturation and differentiation of stem cells. For example, the overexpression of ABCG2 inhibits hematopoietic development. The last, protection from hypoxia appears to be another function of ABC transporters in CSCs[101].

In conclusion, although the mechanisms of cancer stem cell are still unclear, the cancer stem cells must become target of chemotherapy.

### 2.5 Sex hormones and MDR

#### 2.5.1 ER

Estrogens play key roles in development and maintenance of normal sexual and reproductive function. The most potent estrogen produced in the body is 17β-estradiol(E2). Two metabolites of E2, estrone and estriol, although they are high-affinity ligands are much weaker agonists on estrogen receptors (ERs)[102]. Up to now, two type of ERs have been found which named ERα(NR3A1) and ERβ(NR3A2). At the regulation of some genes, particularly those involved in proliferation, ERα and ERβ can have opposite actions [103], a
finding which suggests that the overall proliferative response to E2 is the result of a balance between ERα and ERβ signaling. The expression of ERα is closely associated with breast cancer biology, especially the development of tumors; estrogen hormones induce expression of c-myc and c-fos protooncogenes sufficient for cell division and breast cancer progression[104]. Many studies demonstrated that breast carcinomas which lack ERα expression often reveal more aggressive phenotypes. Furthermore, ERα expression in tumor tissues is a favorable predictor of prognosis in endocrine treatment[105]. ERα typically functions as a transcription factor to regulate specific gene expression which binds to estrogen response elements (ERE) upstream of the target genes. The study of Lisa D. Coles et al. demonstrated that E2 could up-regulate the expression of p-gp in P-gp Overexpressing Cells (NCI-ADR-RES) [106].

Anti-estrogens, designed to block ERα, are widely and effectively used clinically in the treatment of breast cancer. The most common drugs including tamoxifen and toremifene. Some researches show that antiestrogens such as tamoxifen, metabolites of tamoxifen (4-hydroxytamoxifen and N-desmethyltamoxifen), drolxifen, and toremifene stimulated the p-gp ATPase activity and are substrates of p-gp. These results suggest that the antiestrogens may be potent drugs that reverse the multidrug-resistant phenotype mediated by P-gp[107]. However, another study displayed that tamoxifen activates CYP3A4 and MDR-1 genes through steroid and xenobiotic receptor (SXR) in breast cancer cells [108]. But, some other anti-estrogens seem to be more effective on reversing MDR. A study shows that the pure anti-oestrogen ICI 164 could enhance doxorubicin and VBL toxicity to MCF-7/Adr cells 25- and 35-fold, respectively and the pure anti-oestrogens iodotamoxifens completely reversed VBL resistance in the mdr1 transfected lung cancer cell line [109].

Besides affection on p-gp, there are many data showing the relationship between anti-estrogens and BCRP. Imai et al. demonstrated that BCRP mRNA expression was induced by 17b-estradiol in T47D:A18 cells [110]. Our research indicated that BCRP expression is upregulated by 17β-estradiol via a novel pretranscriptional mechanism which might be involved in 17β-estradiol-ER complexes binding to the ERE of BCRP promoter via the classical pathway to activate transcription of the BCRP gene[111]. Besides, we also found that tamoxifen and toremifene could reverse MDR mediated by BCRP in breast cancer cells[104].

### 2.5.2 Progesterone receptor (PR)

Like estrogens, the physiological effects of progesterone are mediated by interaction of the hormone with the progesterone receptor. Up to now, two types of PRs were detected, named as PRA and PRB, respectively. The two PRs are expressed from a single gene as a result of transcription from two alternative promoters[112]. In general, PRB acts as a stronger transcriptional activator, whereas PRA functions as a transcriptional inhibitor of PRB and ER[113]. PR expression in breast cancer is also an important indicator of likely responsiveness to endocrine agents. It has been shown that PRA and PRB are expressed in similar amounts in most breast tumors[114]. Some data indicated that progesterone via PRs may be related to the regulation of MDR in breast cancer. In 1994, Rao US et al found that at 50 microM, progesterone stimulated the P-gp ATPase activity as effectively as verapamil and is a potent drugs inducing p-gp mediated MDR[115]. Recently study displays that transcriptional regulation by E2 and progesterone (P4) likely contributes to the modulation of P-gp levels[116].
Besides that, the relationship between PR and BCRP has also been focus on. Wang et al found there were progesterone response elements on the upstream of BCRP promoter[114] and they note that the identified PRE is exactly the same as the estrogen response element published by Ee et al[117]. They found that PRB is a strong activator of transcription of the BCRP promoter, and PRA represses the PRB activity in the human placental choriocarcinoma BeWo cells. But the real situation in breast cancer may be complex. Because 17β-estradiol can induce PRB expression and down-regulate BCRP expression through posttranscriptional modification[118]; On the other hand, PRA can repress the estrogen receptor activity[113]. So the relationship between progesterone receptor and BCRP needs further data.

2.6 EMT and MDR
Tumor invasiveness, and metastasis, as well as MDR are still great puzzle in the development and treatment of tumors. The interconversion between epithelial and mesenchymal cells (designated as epithelial-mesenchymal or mesenchymal-epithelial transition, EMT or MET, respectively) has received special attention and emerging evidence suggests that epithelial-mesenchymal transitions (EMTs) may take part in the above processes. An epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components[119]. Kalluri R and Weinberg RA divided EMT into three types[119]. Type 1 EMTs can generate mesenchymal cells (primary mesenchyme) that have the potential to subsequently undergo a MET to generate secondary epithelia during implantation, embryogenesis, and organ development. Type 2 EMTs, the program begins as part of a repair-associated event that normally generates fibroblasts and other related cells in order to reconstruct tissues following trauma and inflammatory injury. Type 3 EMTs occur in neoplastic cells that have previously undergone genetic and epigenetic changes, specifically in genes that favor clonal outgrowth and the development of localized tumors. Envidents show that EMT is critically linked with up-regulated invasion, metastasis, and angiogenesis. Figure 1 displays the relationship between EMT and progression of tumors. During the acquisition of EMT characteristics, cells lose epithelial cell–cell junctions, undergo actin cytoskeleton reorganization and decrease in the expression of proteins that promote cell–cell contact such as E-cadherin and β-catenin, and gain in the expression of mesenchymal markers such as vimentin, fibronectin, γ-smooth muscle actin (SMA), N-cadherin as well as increased activity of matrix metalloproteinases (MMPs) like MMP-2, MMP-3 and MMP-9, associated with an invasive phenotype[120]. The modulation of EMT is complicated. Many genes or signal transduction pathways are confirmed to take part in the regulation, such as hepatocyte growth factor (HGF)[121], transforming growth factor beta (TGF-β)[122], epidermal growth factor (EGF)[123], MMP-3[124] and so on. In addition, some transcriptional factors including snail and twist also play important role in EMT[125,126]. Recent studies have shown an intimate relationship between the EMT phenotype and MDR. Kajiyama et al. found that paclitaxel-resistant ovarian cancer cells showed phenotypic changes consistent with EMT[127]. These results were confirmed in other types of tumors.
like gemcitabine-resistant pancreatic cancer cells, oxaliplatin-resistant colorectal cancer cells, lapatinib-resistant breast cancer[120]. In addition, tamoxifen-resistant breast cancer cells undergone EMT with altered β-catenin phosphorylation[128]. It has been indicated that mesenchymal-like cancers might be more sensitive to DNA damaging agents such as doxorubicin, whereas epithelial-like cancers are more sensitive to targeted therapies, such as EGFR and HER2 antagonists [129]. That may be the reason why mesenchymal-like, basal breast cancers are initially more sensitive to chemotherapy than epithelial-like luminal breast cancers[130]. However, it was discussed that basal, mesenchymal-like breast cancers possibly would be more prone to develop drug resistance. So more works need to do to investigate the links of EMT and MDR.

![Fig. 1. The relationship between EMT and progression of tumors. Normal epithelial cells transform to tumor cells. After EMT, tumor cells invade into surrounding normal tissues and distant organs. Then, MET reverse the cells into epithelial cells in the metastasis.](Fig.1.jpg)

2.7 Methylation and MDR

Cancer is known as a genetic disease. Gain, loss, and mutation of genetic information have long been known to contribute to cancer development and progression. It is being increasingly recognized that epigenetic alterations in cancer often serve as potent surrogates for genetic mutations. Methylation of CpG dinucleotides is an important pattern of epigenetics.

Methylation can directly interfere with the binding of transcription factors to inhibit replication and/or methyl-CpG binding proteins that can bind to methylated DNA, as well as regulatory proteins to inhibit transcription[131]. The patterns of CpG methylation are specie and tissue specific. The biological machinery of this system comprises a variety of regulatory proteins including DNA methyltransferases, putative demethylases, methyl-CpG binding proteins, histones modifying enzymes and chromatin remodeling complexes. Alterations in DNA methylation participate in the development of some human diseases, including tumor [132].

Then whether DNA methylation takes part in the modulation of MDR? The answer is yes. El-Osta et al. used inhibitors of DNA methyltransferase (5-azacytidine [5aC]) and histone deacetylase (trichostatin A [TSA]) to examine gene transcription, promoter methylation status, and the chromatin determinants associated with the MDR1 promoter and their result displayed that 5aC and TSA induced DNA demethylation, leading to reactivation of methylated MDR1[133]. Nakayama et al. demonstrated the hypomethylation status of the MDR1 promoter region might be a necessary condition for MDR1 gene overexpression and establishment of P-glycoprotein-mediated multidrug resistance in AML patients[134]. Detailed mapping of MDR1 promoter showed that its promoter is always hypermethylated in drug-sensitive cells, while the drugresistant cells have hypomethylated MDR1.
promoter[135]. Gayatri Sharma et al used methylation-specific PCR to investigate the promoter methylation status of MDR1 in tumor and serum of 100 patients with invasive ductal carcinomas of breast (IDCs) and MDR1 was hypomethylated in 47% tumors and 44% paired serum of IDC patients [136].

The methylation of BCRP has also been focus on. To et al. have shown an active CpG island within the proximal ABCG2 promoter region contributing to inactivation of ABCG2[137]. A follow-up research by Turner et al. demonstrated that ABCG2 expression in multiple myeloma patients and in cell lines is regulated in part by promoter methylation[138].

DNA methylation has been found to anticipate the regulation of other MDR-related genes. Chekhun VF et al. found that the promoter regions of MDR1, GST-pi, genes were highly methylated in MCF-7 cell line but not in its MCF-7/R drug resistant variant. The results suggests that acquirement of doxorubicin resistance of MCF-7 cells is associated with DNA hypomethylation of the promoter regions of the MDR1, GST-pi[139].

3. Strategies to reverse MDR

Since MDR phenomena have been recognised, the war fighting against it has been continuing. Many strategies have been devised to overcome it and mainly divided into three types: modulators, immunotherapy and genetic therapy.

3.1 Modulators of MDR

Because P-gp is the best characterized gene conferring MDR and its wide effects, most modulators target for it. So herein, we divide the modulators into two types: modulators targeting P-gp and targeting other genes.

3.1.1 Modulators of P-gp

Up to now, numerous compounds have been shown to inhibit the drug efflux function of P-gp and therefore, reverse cellular resistance. Since P-gp was first detected in 1976, three generation of modulators are found or synthesis. The process of chemosensitization involves the co-administration of a MDR modulator with an anticancer drug in order to cause enhanced intracellular accumulation via impairing the P-gp function[2].

3.1.1.1 First generation modulators

The first compounds documented to reverse MDR was verapamil (VRP), one of the calcium channel blocker[140]. Studies displayed that VRP enhanced intracellular accumulation of many anticancer drugs, including DOX in numerous cell lines. Subsequent studies revealed that this MDR reversing character is shared by many other calcium channel blockers, clinically available calcium antagonists, and calmodulin antagonists, such as felodipine and trifluoperazine[141]. Indole alkaloids, the anti-malarial quinine and the anti-arrhythmic quinidine, have also been shown to reverse MDR in vitro in experimental cell lines [142]. Cyclosporin A, a commonly used immunosuppressant for organ transplantation, remains one of the most effective first generation of MDR modulators[2].

A number of these first generation MDR modulators, such as VRP and CsA, displayed excellent MDR reversal activities both preclinically and clinically. However, a unique property shared by most first generation modulators is that they are therapeutic agents and typically reverse MDR at concentrations much higher than those required for their
individual therapeutic activity and at these elevated doses, both compounds exhibited severe and sometimes life-threatening toxicities[2].

3.1.1.2 Second generation modulators
In order to solve the high toxicity of the first generation modulators, many newer analogs of the first generation are researched which were more potent and considerably less toxic. Analogs of VRP, including dexverapamil (less cardiotoxic R-enantiomer of VRP), emopamil, gallopamil, and Ro11-2933 (a tiapamil analog) which reversed MDR in vitro to a degree equivalent to VRP, but with marginal toxicity in animal models were documented[2]. The non-immunosuppressive analog of CsA, PSC 833, has demonstrated superior MDR reversal efficacy in conjunction with daunorubicin, DOX, vincristine, vinblastine, taxol, or mitoxantrone in many cell lines in vitro at concentrations of 0.5–2 mM[143]. Although these agents circumvented many of the problems experienced with first generation MDR modulators, when these agents were co-administered with anticancer agents for modulating P-gp-based MDR, they influenced the pharmacokinetics and biodistribution properties of the anticancer drugs, which resulted in increased toxicity to normal organs such as liver and kidney[144].

3.1.1.3 Third generation modulators
The third generation modulators of p-gp have recently been developed using structure–activity relationships and combinatorial chemistry approaches. These agents required low doses (in the nanomolar range (20–100 nM)) to achieve effective reversing concentrations in vivo. The cyclopropyldibenzo-suberane LY 335979 is the representative and is currently under investigation in phase II clinical trials. This substance is highly effective on P-gp-mediated MDR at the concentration of 0.1–0.2μM and shows a very strong affinity for P-gp[145]. Compared to CsA, LY 335979 is characterized by a 10-fold increased potency, latent modulating activity and a blockade specific for P-gp. Another drug the acridonecarboxamide GF 120918 exhibits similar characteristics to LY 335979, but seems to be more effective than LY 335979[146]. The effective concentration of it is 20–100 nM and is one of the most potent and selective MDR modulators disclosed thus far. Both of them are specific for P-gp-mediated MDR since it does not modulate MRP-mediated resistance. In addition, some bispecific chemosensitizers that block both P-gp and MRP were found, such as VX-710 and VX-853[2].

In summary, all the modulators of p-gp can be divided into 10 classifications. Table 1 selected list the modulators of p-gp of each classification reported.

3.1.2 Modulators of other genes
Besides agents targeting P-gp, drugs that inhibit other genes have also been developing. Table 2 displays the selected list of modulators that inhibit other MDR-related genes. Although these agents appear to be well tolerated in combination with anticancer drugs such as DOX, the lack selectivity for the tumor tissue P-GP is still their deficiency which is the cause of adversely affect therapy.

3.2 Immunotherapy of MDR
Another method of MDR reversal is the use of monoclonal antibodies, several of which can inhibit P-gp-mediated drug efflux in vitro. The monoclonal antibody (mAb) MRK16 is the
<table>
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<tr>
<td>MRP1[3]</td>
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<td></td>
<td>XR-9576 (tariquidar)</td>
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<td>VX-710 (bricodar)</td>
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<td>Isothiocyanates</td>
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<td>NSAIDs</td>
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<td>BCRP(ABCG2)[3]</td>
<td>GF-120918 (elacridar)</td>
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<td>GST-pi[150]</td>
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<td>PPMP</td>
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<td>Miglustat</td>
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Table 1. Selected list of P-gp modulators[149]

Table 2. Selected list of modulator targeting other MDR-related genes
first antibody used for reversing MDR by Hamada and Tsuruo[147]. The results found that MRK16 increased intracellular accumulation and cytotoxicity of vincristine and actinomycin D in some MDR cell lines, but had no effect on doxorubicin cytotoxicity. An increase in the accumulation of vincristine and actinomycin D was also observed with two other anti-Pgp mAbs, HYB-241 and HYB-612[148].

3.3 Genetic therapy of MDR

The genetic therapy of MDR mainly includes two methods. The first method was established by Gottesman et al. They produced multidrug resistant bone marrow cells by transfecting them with vectors carrying the MDR1 cDNA and this process allowed bone marrow cells to apply a chemotherapeutic regimen at otherwise unacceptable doses, and thus overcoming MDR[149].

The other method is inhibiting MDR proteins including transcriptional/translational inhibition through the introduction of antisense oligonucleotides or ribozymes or RNA interference. Recently, researchers have done many work targeting different genes, such as mdr1, MRP1, BCRP, GCS and so on. These techniques were proved to have considerable effects on overcoming MDR in vitro and in animal models. However, as many of these methods require gene targeting and transfer, they are unlikely to produce any really significant in vivo applications anytime soon[149].

In summary, although many approaches have been adopted to battle with MDR, it will be for a long time to overcome it completely.

4. References


Targeting New Pathways and Cell Death in Breast Cancer


This book presents novel in interesting find by multiple accomplished investigators in breast cancer. These chapters elucidate new mechanisms of breast cancer cell death as well as discuss new pathways for therapeutic targeting.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:
