MULTIDRUG RESISTANCE IN LOCALLY ADVANCED BREAST CANCER

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

MUSTAFA CAN ATALAY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
THE DEPARTMENT OF BIOTECHNOLOGY

JUNE 2004

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ABSTRACT

MULTIDRUG RESISTANCE IN LOCALLY ADVANCED BREAST CANCER

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June 2004, 70 pages

Breast cancer is the most frequently detected cancer among women. Early diagnosis leads to long term survival when the patients are treated with surgery, radiotherapy, chemotherapy, and hormone therapy. Unfortunately, advanced disease could still be encountered in some patients resulting in a poorer prognosis. The primary treatment modality is chemotherapy for this group of patients. Drug resistance is a serious problem resulting in the use of different drugs during chemotherapy and knowing the possibility of resistance before initiating first line chemotherapy may save time and money, and most importantly, may increase patient's survival. Therefore in this study, multidrug resistance is studied in locally advanced breast cancer patients. The breast tissues obtained from 25 patients both before and after chemotherapy were examined for drug resistance. Reverse transcriptase polymerase chain reaction was used for the detection of *mdr1* and *mrp1* gene expression. In addition, immunohistochemistry technique was used for P-glycoprotein and MRP1 detection. JSB-1 and QCRL-1 monoclonal antibodies were utilized to detect P-glycoprotein and MRP1, respectively.

Five patients were unresponsive to chemotherapy. In four of these patients *mdr1* gene expression was induced by chemotherapy where as the fifth patient initially had *mdr1* gene expression. In addition, Pgp positivity was detected in 9 patients after chemotherapy. Both the induction of *mdr1* gene expression ($p<0.001$) and Pgp positivity ($p<0.001$) during chemotherapy were significantly related with clinical response.

On the other hand, *mrp1* gene expression and MRP1 positivity were detected in 68% of the patients before the therapy. After chemotherapy, *mrp1* expression increased to 84%. Although 80% of the clinically unresponsive patients had *mrp1* gene expression, the relation between *mrp1* expression and clinical drug response was not strong.

Thus, it can be concluded that in locally advanced breast cancer *mdr1* gene expression during chemotherapy contributed to clinical unresponsiveness. However, *mrp1* gene expression did not correlate strongly with the clinical response.

When RT-PCR and immunohistochemistry methods are compared in terms of detection of drug resistance, it seems that both methods gave similar and reliable results.

Key words: Breast cancer, chemotherapy, multidrug resistance, *mdr1*, *mrp1*, Pgp, MRP1, RT-PCR, immunohistochemistry

ÖZ

LOKAL İLERİ EVRE MEME KANSERİNDE ÇOKLU İLAÇ DİRENÇLİLİĞİ

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Haziran 2004, 70 sayfa

Meme kanseri kadınlarda en sık rastlanan kanser türüdür. Erken tanı konulduğunda cerrahi, radyoterapi, kemoterapi ve hormon tedavisi yoluyla hasta uzun süre yaşatılabilir. Ancak bazı hastalarda prognozu daha kötü olan ileri evre hastalık saptanmaktadır. Bu hasta grubunda primer tedavi yöntemi kemoterapidir. İlaç direnci, tedavi sırasında ilaç değişikliği gerektiren ciddi bir sorundur. İlk basamak kemoterapiye başlamadan önce dirençlilik olasılığını bilmek hastanın sağkalımına katkıda bulunacak, zaman ve para tasarrufu sağlayacaktır. Bu nedenle çalışmada lokal ileri evre meme kanseri olan hastalarda çoklu ilaç dirençliliği incelenmiştir. Bu çalışmaya kemoterapi öncesi ve sonrası doku örnekleri alınan 25 hasta dahil edildi. *Mdr1* ve *mrp1* genlerinin ekspresyonlarını saptamak için RT-PCR yöntemi kullanılmıştır. Ayrıca, P-glikoproteini ve MRP1 proteininin varlığını göstermek için immunohistokimya yöntemi kullanılmıştır. P-glikoproteini ve MRP1'i belirlemek için sırasıyla JSB-1 ve QCRL-1 monoklonal antikorları kullanılmıştır.

Kemoterapi sırasında beş hastada ilaçlara klinik olarak yanıt alınamamıştır. Hastalardan dördünde tedavi sonrası *mdr1* gen ekspresyonu indüklenirken, beşinci hastada kemoterapi öncesinde de *mdr1* gen ekspresyonu olduğu belirlenmiştir. Ayrıca, 9 hastada kemoterapi sonrasında Pgp pozitifliği saptanmıştır. Kemoterapi sırasında indüklenen *mdr1* gen ekspresyonu ($p<0.001$) ve Pgp pozitifliği ($p<0.001$) klinik ilaç yanıtıyla istatistiksel olarak ilişkili bulunmuştur.

Diğer taraftan, kemoterapi öncesi *mrp1* gen ekspresyonu ve MRP1 pozitifliği hastaların %68'inde saptanmıştır. Kemoterapiden sonra ise *mrp1* ekspresyonu oranı %84'e yükselmiştir. Klinik yanıtsızlığı olan hastaların %80'inde *mrp1* gen ekspresyonu mevcut olmasına karşın *mrp1* geni ile klinik yanıtsızlık arasında istatistiksel olarak anlamlı bir ilişki bulunamamıştır.

Sonuç olarak, lokal ileri evre meme kanserlerinde kemoterapi sırasında *mdr1* gen ekspresyonunun klinik ilaç yanıtını etkilemekte olduğu söylenebilir. *mrp1* gen ekspresyonunun ise klinik yanıtla güçlü bir ilişkisi bulunmadığı anlaşılmaktadır. Ayrıca, RT-PCR ve immünhistokimya yöntemlerinin ilaç dirençliliğini belirleme bakımından birbirleriyle uyumlu ve güvenilir sonuçlar verdiği belirlenmiştir.

Anahtar kelimeler: Meme kanseri, kemoterapi, çoklu ilaç dirençliliği, *mdr1*, *mrp1*, Pgp, MRP1, RT-PCR, immünhistokimya

ACKNOWLEDGEMENTS

I would like to express my special thanks to my supervisor Prof. Dr. Ufuk GÜNDÜZ for providing me her guidance, support, and insight at every stage during my research.

I would like to thank to Assoc. Prof. İsmet GÜRHAN as my co-supervisor and to Prof. Dr. Ahmet DEMİRKAZIK and Prof. Dr. Nesrin HASIRCI for their valuable contributions to my research through the years. I would also appreciate the contributions of Prof. Dr. Hüseyin Avni ÖKTEM and Prof. Dr. Fikret ARPACI as members of the Examining Committee to this study.

I would like to thank my wife and daughter for their patience and support through all those years of study.

I also appreciate the helps of the valuable members of our laboratory. My special thanks goes to D. Ogan ABAAN and Dr. Çiğdem IRKKAN for their technical support during the experiments.

I also thank to METU Research Fund for financially supporting the study with Project No: 2000-07-02-00-23.

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ABBREVIATIONS

ABC	ATP binding cassette
β_2M	Beta-2-microglobulin
BCRP	Breast cancer resistance protein
DCIS	Ductal carcinoma *in situ*
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
GSH	Glutathione
GTC	Guanidium thiocyanate
IHC	Immunohistochemistry
LABC	Locally advanced breast cancer
LCIS	Lobular carcinoma *in situ*
LTC4	Leukotriene C_4
LRP	Lung cancer resistance-related protein
MDR	Multidrug resistance
M-MuLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MOPS	3-(N-Morpholino)propanesulsonic acid
MRP	Multidrug resistance-associated protein
NBD	Nucleotide binding domain
PBC	Primary breast cancer
PBS	Phosphate buffered saline
Pgp	P-glycoprotein
PMEA	9-(2-phosphonylmethoxyethyl) adenine
RT-PCR	Reverse transcriptase polymerase chain reaction
TMD	Transmembrane domain

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is one of the most important health problems of the current era and also a leading cause of death among populations. Cancer can simply be defined as unregulated cell division leading to a tumor formation in any part of the body. In its natural course, tumor mass continues to grow invading the surrounding tissues and finally tumor cells get access to the lymphatic and vascular systems spreading to distant organs which results in metastasis. In order to be successful in the treatment of cancer, early diagnosis, before the tumor spreads to the surrounding tissues or distant organs, is mandatory.

It is now known that most cancer types result from accumulation of multiple errors in DNA that may affect primarily the regulatory pathways in the cell. Although the currently used treatment modalities are mainly directed to the macroscopic destruction of the tumor mass, the presence of systemic dissemination could not be denied and systemic treatments are widely used in order to control the microscopic disease. Despite these advances in treatment, the development of new strategies towards the correction of molecular impairments in the cell is indispensable.

1.2 Breast Cancer

Breast cancer is the most commonly diagnosed malignancy in women around the world, especially in the Western countries. It accounts for almost one fifth of deaths caused by

cancer (Winer EP *et al.*, 2001). Among US women, the lifetime risk for being diagnosed with breast cancer is 1 in 8 (Ahearne PM *et al.*, 1999). These figures are clear enough to indicate that breast cancer is an important health issue for women as well as the society. Strategies to reduce the morbidity and mortality of this enormous health issue include the identification and modification of the risk factors, earlier diagnosis and treatment, and improved treatment strategies. Physician's role in this environment should be to diagnose the disease as early as possible to definitely cure the patients. Screening programs are used worlwide to achieve this goal. Even though screening programs and the education of the public have lead to the higher number of patients diagnosed at early stages of the disease, about 5-20% of patients still have a locally advanced breast cancer at admission (Hortobagyi GN, 1994). Besides, the number of patients with advanced disease tends to increase in populations with limited access to health resources and this leads to further difficulties in the treatment of breast cancer. Patients with advanced disease further complicate the treatment strategies. The need for the development of new drugs increase due to observed drug resistance in these patients.

1.2.1 Histopathologic Types of Breast Cancer

Breast cancer originates from the terminal ducto-lobular unit of breast tissue. Breast cancer that has not invaded the basement membrane and thus confined within the terminal ducto-lobular units is termed carcinoma in-situ. Mainly, there are two types of in-situ cancers; lobular carcinoma in-situ and ductal carcinoma in-situ.

On the other hand, breast cancers that invade the basement membrane are called invasive cancers. The two main types are synonymously called invasive lobular and ductal carcinoma. The main difference between in-situ and invasive cancers is the ability of the invasive forms to spread through the lymphatic and vascular vessels located under basement membrane leading to regional lymphatic and distant organ metastases.

1.2.1.1 Lobular Carcinoma In-Situ

Lobular carcinoma in-situ (LCIS) originates from the lobular elements of the breast. Thus, it is only observed in women since men have no lobular units in their breasts. LCIS is usually not detectable macroscopically during physical examination and is frequently an incidental microscopic finding in the breast tissue removed for another reason. In the biopsy specimens done for benign breast abnormalities, it was found that only 0.5% to 3.6% are LCIS (Winer

EP *et al.*, 2001). It is noted to be more common in younger women with 80% to 90% of cases of LCIS occurring in premenopausal women (Winer EP *et al.*, 2001). Besides, LCIS is accepted as a premalignant lesion of the breast since an invasive cancer could develop in any part of the breast and even in the contralateral breast after the diagnosis of LCIS. Bilaterality and multicentricity are common features of LCIS.

1.2.1.2 Ductal Carcinoma In-Situ

Ductal carcinoma in-situ (DCIS) is an entity distinct in both its clinical presentation and its biologic potential from LCIS. DCIS originates from the ductal epithelium in the breast and could be diagnosed in both males and females. These are the precursor lesions for invasive ductal carcinoma and during its natural course, it changes into its invasive form. DCIS is characterized pathologically by a proliferation of presumably malignant epithelial cells within the mammary ductal-lobular system without light microscopic evidence of invasion into the surrounding stroma. However, DCIS encompasses a heterogeneous group of pathologic lesions that differ in their growth pattern and cytologic features. The traditional system for classifying DCIS is based primarily on architectural pattern and grouped into five major subtypes: comedo, cribriform, micropapillary, papillary, and solid (Winer EP *et al.*, 2001). The comedo type usually appears more malignant cytologically and is more often associated with invasion than are the other types.

1.2.1.3 Invasive Lobular Carcinoma

These carcinomas originate in terminal ductules of the lobule and possess characteristic features that distinguish them from the lesions of larger ducts. Invasive lobular carcinoma constitutes approximately 10% of breast cancers. When compared to invasive ductal carcinoma, they usually have a propensity for bilaterality, multicentricity, and multifocality. The treatment strategies utilized are similar to those used for invasive ductal carcinoma. Furthermore, the stage of the disease is the major determinant of outcome rather than the histologic type of the tumor. Thus, at the same stage both invasive ductal and lobular carcinomas have similar prognosis.

1.2.1.4 Invasive Ductal Carcinoma

Invasive ductal carcinoma is the most common type of invasive breast cancer accounting for 75-80% of cases. These lesions are usually single and unilateral. Invasive ductal carcinomas have similar prognosis as invasive lobular carcinoma when diagnosed at the same stage.

1.2.1.5 Other Invasive Types

Besides the above mentioned common types of invasive breast cancers, there are other rare forms such as medullary, papillary, mucinous, tubular, apocrine and adenoid cystic carcinoma (Winer EP *et al.*, 2001). These histologic types usually have better biological properties rendering them to be known as less aggressive types of breast cancer. The slower progression of disease in these types results in a better prognosis for patients.

1.2.2 Staging of Breast Cancer

Staging is an important issue for all types of cancers and it enables us to group the patients according to almost equal suvival probabilities. Besides, staging standardizes the patients in different studies and makes an easier comparison possible. As in the case of most of the cancers, staging of breast cancer takes into consideration the size of the tumor (T), the number and location of metastatic lymph nodes (N), and distant organ metastasis (M). According to TNM staging system (Greene FL *et al.*, 2002), breast cancer patients are divided into stages I to IV. Stages I and II are called as early stages while stage III designates locally advanced breast cancer. Stage I includes patients with tumor size < 2 cm without any lymph node or distant metastases where as stage II patients have tumors < 5 cm with axillary lymph node metastases and without distant metastases. The definition of locally advanced breast cancer is variable. Patients with inoperable stage III B disease are always included where as patients with stage III A disease or supraclavicular lymph node metastases or even stage IIB patients are included in locally advanced patient group in different studies. Among the locally advanced stage patients, stage IIIA constitutes the patients with tumors < 5 cm, have spread to the axillary lymph nodes which are attached to each other or with tumors > 5 cm and spread to the axillary lymph nodes where as stage IIIB includes the patients with either the skin or underlying muscle invasion, usually with edema or ulceration of the skin. Since the systemic spread of the disease is a source of concern for the locally advanced breast cancer patients, systemic chemotherapy is used as the primary treatment modality.

The term locally advanced breast cancer includes a heterogeneous group of patients including those with neglected, slow-growing tumors as well as those with biologically aggressive disease. Locally advanced breast cancer is a relatively uncommon presentation of breast cancer in developed countries accounting for 5-20% of cases (Hortobagyi GN, 1994). However, in other countries, locally advanced breast cancer is more common accounting for almost half of the cases. This difference could be due to variations in public awareness and attitudes, as well as the availibility of medical resources such as screening mammography.

1.2.3 Treatment of Breast Cancer

Treatment of breast cancer mainly consists of local and systemic therapies. Surgery and radiotherapy are the treatment modalities used for local control of the disease where as chemotherapy and hormonotherapy are used for systemic control of the disease. The properties of the patient and tumor determine the choice of treatment. Although the indications to use them differ due to histopathologic characteristics of the tumor, the best results are obtained when these treatment methods are applied in combination.

1.2.3.1 Surgery

Surgery is the mainstay in the treatment of breast cancer and has evolved from more radical methods such as radical mastectomy to conservative ones in time. The aim of surgery is to remove all of the tumor tissue with microscopically negative margins and include the regional lymph nodes in the specimen. Currently, the most commonly used surgical methods are modified radical mastectomy and breast conserving surgery with axillary dissection (Winer EP *et al.*, 2001). In modified radical mastectomy, all of the breast tissue is removed with axillary lymph nodes. In contrast, only the tumor tissue with surrounding rim of normal breast tissue is removed for breast conservation including regional lymph nodes. As more conservative surgical approaches are preferred in breast cancer, sentinel lymph node biopsy has been a new alternative method for axillary dissection. This method is utilized with the aim of decreasing the complication rate of axillary dissection and will probably be more frequently used in the future.

In contrast, surgery cannot be used primarily in most locally advanced breast cancer patients (Hortobagyi GN *et al.*, 1988). The reasons for this are inability to achieve negative surgical margins even after removing the whole breast tissue due to large tumor size and the higher probability of systemic spread of the disease. Thus, surgery is used after induction

chemotherapy depending on the response of the patient to the drugs. The clinical response of the patient is an important determinant of further surgery and survival of the patient.

1.2.3.2 Radiotherapy

Radiotherapy is the other treatment method used for local control of breast cancer and is usually given after surgery. Postoperative radiotherapy improves local control and probably survival of the patient (Winer EP *et al.*, 2001). Patients with four or more positive lymph nodes in the axilla or tumors >5 cm receive postoperative radiotherapy to improve local control. There may also be a survival benefit in these patients. The data regarding patient selection for survival advantage are less clear, but the most recent evidence suggests that the greatest survival benefit is seen in node-positive patients with low tumor burdens, such as patients with fewer positive nodes or smaller tumors (Ahearne PM *et al.*, 1999). Radiation therapy in these patients for survival benefit is worthy of consideration pending more definitive data.

The role of radiotherapy in locally advanced breast cancer differs in certain aspects. It may be applied after induction chemotherapy if an adequate response is not obtained. Radiotherapy may further decrease the tumor size making it possible to perform surgery. In addition, radiotherapy is an indispensable part of treatment after surgery for locally advanced breast cancer patients (Hortobagyi GN *et al.*, 1988).

1.2.3.3 Chemotherapy

Current concepts on the natural course of breast cancer support that occult metastases are commonly present even in patients with early breast cancer (Ahearne PM *et al.*, 1999). This view is based on the fact that, even following effective local treatment, many patients develop metastatic involvement over time and improvements in local control have been shown to provide only a small decrease in distant metastases. Given this, improving the long-term outlook for newly diagnosed breast cancer patients with early-stage disease can only be accomplished with improvements in systemic therapy. Clinical trials have demonstrated significant improvements in survival for patients treated with chemotherapy compared with controls. Chemotherapy could be given to the patients either before (neoadjuvant) or after surgery (adjuvant). Adjuvant or neoadjuvant chemotherapy, hormonal therapy, or both are now in widespread use around the world. Since its introduction, there has been a decrease in the death rate from breast cancer, suggesting a beneficial effect.

Systemic treatment of breast cancer with chemotherapy initially consisted of single drugs (Winer EP *et al.*, 2001). Later, improved results were obtained with combination chemotherapy. A protocol composed of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) was one of the early combinations with proven efficacy (Bonadonna G *et al.*, 1995). With the inclusion of anthracyclines in chemotherapy protocols, they became the most widely used drugs in the treatment of breast cancer. Anthracyclines show their effects by inducing formation of covalent topoisomerase II – DNA bound complexes that results in single or double strand breaks. Chemotherapy regimens containing various anthracyclines such as 5-fluorouracil, doxorubicin and cyclophosphamide (FAC) and 5-fluorouracil, epirubicin and cyclophosphamide (FEC) are used both in the adjuvant and neoadjuvant settings of breast cancer treatment. These two regimens are often preferred as the first line chemotherapeutics in the treatment of locally advanced breast cancer.

Historical results in treating patients with locally advanced breast cancer using surgery and radiotherapy were uniformly poor and more aggressive local treatments increased complications without improving survival rates. However, with the development of effective chemotherapy, most cases of inoperable breast cancer could be operated safely (Hortobagyi GN *et al.*, 1988). Besides, systemic therapy decreased the risk of systemic recurrence and the absolute benefit for this group of patients increased.

The response rates to neoadjuvant chemotherapy are high, possibly due to the presence of an intact blood supply. The advantages of using neoadjuvant chemotherapy could be as follows (Winer EP *et al.*, 2001);

(1) the early initiation of systemic therapy in patients with a high probability of distant failure,

(2) the opportunity to directly assess the response to chemotherapy,

(3) a reduction in the extent of surgery needed to render the patients grossly free of disease.

If the tumor does not respond to the initial chemotherapy regimen, substitution with other drugs or the timely initiation of radiotherapy may be beneficial. Clinical response is usually determined by physical examination, but can be supplemented by mammography and/or ultrasonography. It is important to note, however, that clinical response does not always correlate with pathologic response (Herrada J *et al.*, 1997). Up to one-third of patients found to be histologically free of disease have residual abnormalities by palpation or imaging after induction chemotherapy. Conversely, approximately one-third of patients thought to be in complete remission on clinical grounds still have residual disease on pathologic examination.

Pathologic response is the best indicator of response to treatment, and patients with a complete pathologic response have a favorable prognosis (Trecate G *et al.*, 1999).

A wide variety of chemotherapy regimens have been used as neoadjuvant treatment, with most incorporating anthracyclines. These regimens generally produce response rates in at least two-thirds of patients with a complete pathologic remission rate of approximately 10-20% (Winer EP *et al.*, 2001). There is no evidence that one regimen is better than another; neoadjuvant treatment is generally given to maximal response. The optimal duration and sequencing of preoperative and postoperative therapy has not been determined as well. Attempts are under way to determine if there are molecular predictors of response to neoadjuvant therapies. Clinical resistance to chemotherapy drugs is an important issue leading to loss of time and money. Multidrug resistance mechanisms may have a role in clinical resistance to chemotherapy and worth investigating.

Following successful neoadjuvant chemotherapy, a variety of options for local treatment have been investigated. Surgery following neoadjuvant chemotherapy permits a pathologic assessment of response, the rapid reintroduction of chemotherapy, and the use of lower doses of radiotherapy. The most typical approach is to use an anthracycline-containing chemotherapy as induction, followed by modified radical mastectomy, additional chemotherapy, and then consolidative radiotherapy (Winer EP *et al.*, 2001). In general, prognostic factors predictive of good outcome in patients with locally advanced breast cancer are the same as in lower stages of primary breast cancer; namely, smaller tumor size, slower growth rate, better differentiation, and fewer involved axillary lymph nodes. In addition to these classical factors, the response of locally advanced cancers to preoperative chemotherapy is an additional important prognostic factor. As noted, evidence from many series indicates that patients with rapidly responding cancers and those who achieve a complete remission have a better outcome than patients who do not have a good response to chemotherapy.

1.2.3.4 Hormone Therapy

Hormone therapy is another method used for systemic control of breast cancer. It is usually used in addition to chemotherapy. Until now, tamoxifen is the most widely studied drug. Tamoxifen was initially considered a promising candidate for adjuvant treatment because of its efficacy against advanced disease and relative lack of toxicity (Winer EP *et al.*, 2001). The benefits of tamoxifen are substantial and are seen in both premenopausal and

postmenopausal women. Tamoxifen, taken for approximately 5 years, reduces the annual odds of disease recurrence by 47% and the annual odds of death by 26% (Early Breast Cancer Trialists' Collaborative Group, 1998). The degree of benefit is similar in younger and older women. The benefits of tamoxifen administered for 5 years were similar despite the presence or absence of chemotherapy. Importantly, the benefits seen with tamoxifen were only seen in women with ER-positive tumors. There appeared to be no benefit from adjuvant tamoxifen in ER-negative patients in terms of either recurrence or death. Tamoxifen lowers the risk of disease recurrence even after it has been discontinued (Early Breast Cancer Trialists' Collaborative Group, 1998)

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1.3 Drug Resistance

Chemotherapy, as mentioned previously, is the main systemic treatment modality prolonging the survival rates in breast cancer. However, not all of the patients could be cured with chemotherapy and the response rates of the patients differ due to various drug resistance mechanisms (Paul and Cowan, 1999). The possible mechanisms of drug resistance are given in Table 1.1. These mechanisms act either alone or in combination preventing a definite clinical response. Some of these mechanisms play a role against specific drugs where as others result in the development of non-specific drug resistance.

1.4 Multidrug Resistance

Multidrug resistance is a significant challenge in the treatment of cancer. This type of resistance develops against various "naturally occuring" drugs with different structure and mechanism of action such as anthracyclines, taxanes, epipodophyllotoxins, and vinca alkaloids (Ambudkar SV *et al.*, 2003). However, these drugs do share some features in that they are all soluble in lipids, enter the cells by passive diffusion, and are relatively large in size (300-900 Mr).

Table 1.1: General mechanisms of drug resistance (Paul and Cowan, 1999)

I. Cellular and Biochemical Mechanisms
Decreased Drug Accumulation
Decreased drug influx
Increased drug efflux
Altered intracellular distribution of the drug
Altered drug metabolism
Decreased drug activation
Increased inactivation of drug/toxic intermediate
Increased Repair of Drug Induced Damage
Altered Drug Targets, Qualitative and Quantitative
Altered Cofactor of Metabolite Level
Decreased Apoptosis
II. Mechanisms Relevant *in vivo*
Host-drug Interactions
Increased drug interactions by normal tissues
Decreased drug activation by normal tissues
Relative increase in normal tissue drug sensitivity/toxicity
Host-tumor Interactions

Tumor cells that are initially sensitive to a broad range of drugs can frequently develop resistance to a group of anti-cancer drugs. This resistance is mainly due to the increased drug efflux driven by pumps located at the cell membrane. Drug accumulation in the cell occurs basically by two mechanisms. Water-soluble, hydrophilic drugs such as cisplatin and anti-folates cross the cell membrane with the help of transporters or hydrophilic channels. Individual mutations in these carriers result in single-agent resistance (Ambudkar SV *et al.*, 2003). In contrast, hydrophobic drugs, such as natural drugs, enter the cell by diffusion across the plasma membrane. Energy-dependent transport systems are needed to expel these drugs out of the cell. These pumps, namely adenosine triphosphate (ATP) binding cassette (ABC) transporters work by coupling the hydrolysis of ATP to substrate transport across the cell membrane (Chang G, 2003). Today, 48 members of the ABC transporter family have been identified and most of them are located on the cell membrane translocating drugs,

xenobiotics, endogenous substances or ions to various compartments. ABC transporters have been divided into seven different classes (A-G) based on sequence similarities. Members of four of these classes (A, B, C and G) have been previously shown to cause drug resistance on cultured cells (Gottesman MM, 2002).

The members of ABC transporters family have common features in their structures (Ejendal and Hrycyna, 2002). All ABC transporters are composed minimally of two nucleotide binding domains (NBD) and two transmembrane domains (TMD) except breast cancer resistance protein (BCRP or ABC G2) which has one TMD and one NBD and probably functions as a dimer. NBD is the part of these transporters that bind and hydrolyze ATP and TMDs are those parts of the molecule specific for the substrate binding and determine the main difference between various members of the family.

Until now, the highly recognized members of this family of proteins are P-glycoprotein (Pgp) and multidrug resistance associated protein (MRP) (Chen CJ *et al.*, 1986; Cole SP *et al.*, 1992). In addition to these proteins, breast cancer resistance protein (BCRP) has been identified and been a subject for recent studies (Doyle LA *et al.*, 1998). Tissue localization, natural and drug substrates of ABC transporters are shown in Table 1.2.

1.5 P-glycoprotein

Pgp was the first of the human ABC transporters to be described and the most widely studied (Chen CJ *et al.*, 1986). It is encoded by the multidrug resistance gene 1 (*mdr1*) which comprises of 28 exons and is located on 7. chromosome. Pgp is a typical ABC transporter protein that is 190 kDa and composed of 1280 amino acids. It has two homologous halves, each containing a TMD and an ATP-binding domain. The schematic structure of Pgp is shown in Fig 1.1. Pgp functions as an efflux pump hydrolizing ATP and is responsible for the transport of toxic substances and secreted molecules. The substrates and modulators of Pgp pump are depicted in Table 1.3. In the body, Pgp is commonly found in the organs with secretory functions such as liver, kidneys, intestines, and bile ducts. In contrast, Pgp is not present in breast tissue (Ambudkar SV *et al.*, 2003). In addition, Pgp plays a protective role in blood-brain barrier preventing the penetration of toxic substances to cerebrospinal fluid by expelling them out of the cells (Cordon-Cardo C *et al.*, 1989).

Table 1.2 Tissue localization and substrates of ABC transporters

Common/ Systematic Name	Tissue	Drug Substrates	Natural Substrates
Pgp / MDR1 (ABCB1)	Intestine, liver, kidney, placenta, blood-brain barrier	Anthracyclines, vinca alkaloids, taxanes, epipodophylotoxins	Neutral and cationic organic compounds
MDR2 (ABCB4)	Liver	Paclitaxel, vinblastine	Phosphatidylcholine
MRP1 (ABCC1)	All tissues	Anthracyclines, vincristine, etoposide, methotrexate	Glutathione and other conjugates, organic anions
MRP2 (ABCC2)	Liver, kidney, intestine	Methotrexate, etoposide, doxorubicin, cisplatin, vincristine	Similar to MRP1, non-bile salt organic anions
MRP3 (ABCC3)	Liver, kidney, intestine, pancreas, adrenal	Methotrexate, etoposide, doxorubicin, cisplatin, vincristine	Glucuronate and glutathione conjugates, bile acids
MRP4 (ABCC4)	Prostate, testis, ovary, pancreas	Methotrexate, thiopurines	Nucleoside analogues, organic anions
MRP5 (ABCC5)	Most tissues	6-Mercaptopurine, 6-Thioguanine	Nucleoside analogues, organic anions, cyclic nucleotides
MRP6 (ABCC6)	Liver, kidney	Unknown	Anionic cyclic pentapeptide
MRP7 (ABCC10)	Liver, heart, kidney	Unknown	Glutathione conjugates, lipophilic anions
BCRP (ABCG2)	Placenta, intestine, breast, liver	Anthracyclines, topotecan, mitoxantrone	Prazosin

(Ambudkar SV *et al.*, 2003)

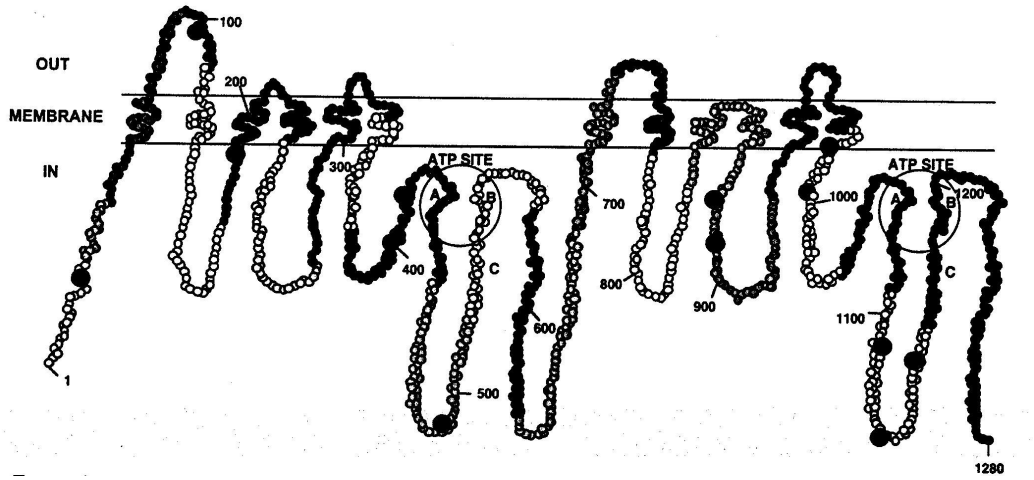

Fig. 1.1. Schematic structure of P-glycoprotein depicting transmembrane domains and ATP-binding sites (Ambudkar SV *et al.*, 2003).

In patients with cancer, during chemotherapy, Pgp functions as a drug pump extruding drug molecules through the plasma membrane. Uncharged drug molecules entering the cell by passive diffusion are removed from the membrane before they can enter the cell. This drug transport is achieved through a channel formed by Pgp molecules. Previous studies have shown an increased *mdr1* gene expression in various cancers including colorectal, renal, breast, and ovarian cancers (Ambudkar SV *et al.*, 2003).

Table 1.3 Selected substrates and modulators of P-glycoprotein

Substrates	Modulators
Vinca alkaloids	*Calcium channel blockers*
Vinblastine, vincristine	Verapamil, dihydropyridines
Anthracyclines	*Antihypertensives*
Daunorubicin, doxorubicin, epirubicin	Reserpine
Antibiotics	*Immunosuppressants*
Actinomycin D	Cyclosporin A
Other cytotoxic drugs	*Steroid hormones*
Mitomycin, paclitaxel	*HIV protease inhibitors*

1.6 Multidrug Resistance Associated Protein Family

Multidrug resistance associated proteins (MRP) are members of ABC transporter protein family. Although they resemble other members of this family, some of MRPs has a third TMD composed of five transmembrane helices, an extra intracellular loop and extracellular N-terminus distinguishing them from other proteins (Kruh and Belinsky, 2003). The schematic representation of the selected members of MRP family with different structures are shown in Fig. 1.2. In normal tissues, MRPs are located in the cytoplasm functioning as carriers of various molecules to the organelles (Flens MJ *et al.*, 1996). In contrast, MRPs are known to be located both in the cytoplasm and on the cellular membrane in cancer cells (Flens MJ *et al.*, 1994). Upto date nine members of this family have been identified in the previous studies (Kool M *et al.*, 1997; Kool M *et al.*, 1999; Schuetz JD *et al.*, 1999; Hopper E *et al.*, 2001). The substrates, physiological functions, and resistance profile of this group of proteins are depicted in Table 1.4.

MRP1 is the founder of a family of proteins and is 170 kDa consisting of 1531 amino acids. The amino acid sequence has 15% similarity to Pgp. MRP1 is coded by *mrp1* gene located on 16. chromosome. It functions as a glutathione and glucuronate conjugate pump and confers resistance against drugs such as anthracyclines, epipodophyllotoxins, vinca alkaloids, camptothecins, and methotrexate. In contrast to Pgp, resistance to taxanes has not been reported for MRP1 (Cole SP *et al.*, 1994; Chen ZS *et al.*, 1999). Numerous reports have documented the expression of *mrp1* in cancers that are treated with anthracyclines, camptothecins, and etoposide such as leukemia, breast, and colorectal cancers (Burger H *et al.*, 2003). For this reason, it is possible to infer that *mrp1* contributes to the inherent sensitivity of cancers in which it is expressed. MRP1 transports lipophilic anions and functions as a basolateral transporter and moves compounds away from luminal surfaces and into tissues below the basement membrane.

MRP2 functions as a canalicular efflux pump for amphipathic anions and has a role in resistance against anti-cancer drugs (Kruh and Belinsky, 2003). Previously, MRP2 was referred as the canalicular multispecific organic anion transporter. The substrate selectivity of MRP2 resembles that of MRP1 with respect to glutathione and glucuronate conjugates. Besides, it is functionally similar to Pgp in its involvement in the elimination of toxic compounds and its role as a barrier in gut and placenta. In addition, MRP2 has a role in Dubin-Johnson syndrome indicating its function in the excretion of bilirubin glucuronide (Jedlitschky G *et al.*, 1997). Although its significance as an *in vivo* resistance factor remains

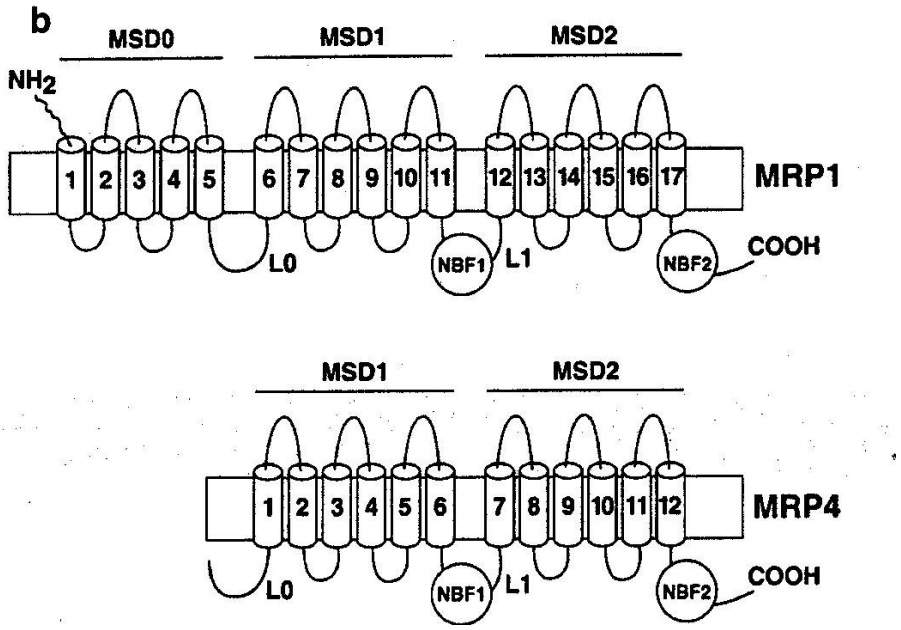

Fig. 1.2. Schematic structure of multidrug resistance associated proteins (Kruh and Belinsky, 2003).

Table 1.4 Properties of MRP family members (Kruh and Belinsky, 2003)

Protein	Conjugates	Glutathione	Resistance Profile	Substrates
MRP1	+	+	Anthracyclines, Vincristin, Etoposide, Irinotecan, Methotrexate	LTC4
MRP2	+	+	Anthracyclines, Vincristin, Etoposide, Irinotecan, Methotrexate, Platinum derivatives	Bilirubin glucuronide
MRP3	+	--	Etoposide, Methotrexate	Glycocholic acid
MRP4	+	+	6-Mercaptopurine,Methotrexate,PMEA	Cyclic nucleotides
MRP5	--	+	6-Mercaptopurine, PMEA	Cyclic nucleotides
MRP6	+	?	Anthracyclines, Etoposide Platinum derivatives	?
MRP7	+	?	?	?
MRP8	?	?	5-FU, PMEA	Cyclic nucleotides

to be determined, expression of *mrp2* has been reported for several types of cancers including colorectal, breast, ovary cancers, and leukemia (Burger H *et al.*, 2003).

MRP3 has the highest degree of structural resemblance to MRP1 (58%) and its substrate selectivity overlaps with that of MRP1 and MRP2 with respect to the transport of glutathione and glucuronide conjugates. However, its role in drug resistance is limited due to its lower affinity to glutathione.

MRP4 and MRP5 are organic anion transporters and mainly mediate the transport of cyclic nucleotides such as cAMP and cGMP. Similarly, MRP8 is thought to have a role in the extrusion of cyclic nucleotides. For this reason, these three members of MRP family confer resistance to certain nucleotide analogs such as 6-mercaptopurine, 6-thioguanine and methotrexate, instead of natural products.

MRP6 was discovered as a molecule whose genetic deficiency leads to a rare autosomally inherited connective tissue disease, namely pseudoxanthoma elasticum. MRP6 is able to transport lipophilic anions. Besides, MRP6 can function as a drug pump resulting in low levels of resistance to etoposide, anthracyclines, and cisplatin.

MRP7 and MRP9 are the most recently described family members. The functional studies are still going on for these two new molecules.

1.7 Multidrug Resistance Mechanisms in Breast Cancer

In previous studies related to *mdr1* gene expression in breast cancer patients, a positivity rate changing between 0-100% was reported (Wallner J *et al.*, 1991; Merkel DE *et al.*, 1989; Goldstein LJ *et al.*, 1989; Keith and Brown, 1990; Kacinski BM *et al.*, 1989; Chevillard S *et al.*, 1997; Charpin C *et al.*, 1994; Wang CS *et al.*, 1997; Faneyte IF *et al.*, 2001; Lacave R *et al.*, 1998; Dexter DW *et al.*, 1998; Filipits M *et al.*, 1996; Arnal M *et al.*, 2000). Merkel (1989) detected no *mdr1* gene expression in breast cancer patients where as other studies including primary breast cancer patients found *mdr1* gene expression ratio changing between 16-56% (Goldstein LJ *et al.*, 1989; Keith and Brown, 1990; Kacinski BM *et al.*, 1989; Wallner J *et al.*, 1991). This discrepancy in the results may be due to the use of different methods in the detection of gene expression such as RT-PCR, Northern blot, or in-situ hybridization. As the sensitivity of the method used increased, the detected *mdr1* gene

expression also increased. RT-PCR based studies reported *mdr1* gene expression as 60-100% of the breast cancer patients (Charpin C *et al.*, 1994; Wang CS *et al.*, 1997; Dexter DW *et al.*, 1998; Filipits M *et al.*, 1996; Arnal M *et al.*, 2000).

On the other hand, *mrp1* positivity was detected in 70-100% of breast cancer patients using RT-PCR method in the previous studies (Dexter DW *et al.*, 1998; Filipits M *et al.*, 1996). Similar to the results of gene studies, by immunohistochemical techniques the protein products, Pgp and MRP1, were detected in 0-100% of the breast cancer patients (Faneyte IF *et al.*, 2001; Ro J *et al*., 1990; Vargas-Roig LM *et al.*, 1999; Rudas M *et al.*, 2003; Nooter K *et al.*, 1997; Linn SC *et al.*, 1997). The differences in the detected protein products between the studies may be due to either the heterogenous group of patients studied or different monoclonal antibodies used for staining.

Besides Pgp and MRP1, other proteins also have a role in drug resistance in breast cancer patients. Lung cancer resistance-related protein (LRP) is another membrane pump involved in MDR. LRP is a 110 kDa protein and its related gene is located on chromosome 16 near *mrp1* gene (Scheffer GL *et al.*, 1995). LRP is the major vault protein in the cell functioning as a pump between cell nucleus and cytoplasm. Tissue distribution of LRP was reported to be similar to MRP1 (Izquierdo MA *et al.*, 1996). Studies evaluating its function in breast cancer patients are rare, but LRP expression is detected in MCF-7 breast cancer cell lines (Beck J *et al.*, 1998).

Breast cancer resistance protein (BCRP) is another recently discovered protein involved in drug resistance (Doyle LA *et al.*, 1998). Although it has been shown to confer resistance to various drugs in cell cultures, its role in cancer patients has yet to be defined (Doyle and Ross, 2003)

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1.8 Reversal of Multidrug Resistance

Since the initial description of MDR phenotype and the discovery of related membrane proteins, attempts to reverse or “modulate” MDR had been made in previous studies (Aszalos A *et al.*, 1999; Evers R *et al.*, 2000a; Evers R *et al.*, 2000b). There are several reasons that justify a search for pharmacologic agents capable of modulating MDR. Usually, MDR related genes are highly expressed in various clinically resistant tumors and in some cases these genes appear to be activated after chemotherapy suggesting a survival

mechanism for the tumor cells. On the other hand, selection of MDR phenotype cells during chemotherapy may be the reason for a clinically resistant tumor.

Until today, various pharmacologic agents have been reported to be transport substrates of MDR related proteins (Aszalos A *et al.*, 1999; Evers R *et al.*, 2000b). These drugs act by competitively inhibiting the MDR pumps and sensitizing the cells to chemotherapy drugs. Among these inhibitors of drug pumps are verapamil, cyclosporin, nifedipine, quinidine, and reserpine (Böhme M *et al.*, 1993; Lum BL *et al.*, 1993). Many new drugs are under development. These drugs, when combined with the chemotherapy drugs, result in a better response. However, as the efficacy of the chemotherapeutics increase, toxic side effects show a similar increase and dose adjustments will probably be needed in clinical use.

1.9 Tumor Suppressor Genes, Oncogenes and Steroid Receptors in Breast Cancer

Many oncogenes are overexpressed and various tumor suppressor genes are mutated in different cancer cells. These genes have a variety of functions in the cancer cell interfering with normal functions of the cell. In breast cancer, p53, p21, and p27 tumor suppressor genes, c-erb-b2, an epidermal growth factor, BRCA1 and BRCA2 genes are known to be mutated in patients.

p53 has been reported to be mutated in various cancers including breast cancer. Mutated p53 was found in almost 50% of breast cancers (Ziyaie D, *et al.*, 2000). In mutated form its conformation changes, stability increases, and its regulatory functions are altered. Wild-type p53 appears to function in the context of DNA damage pausing the cell cycle to allow time to the cell for DNA repair. p53 achieves this by triggering a check-point that blocks further progression in the cell cycle. This check-point works through inducing p21 and p27, inhibitors of cdk-cyclin kinases. In case of severe damage to DNA, apoptosis is induced by p53 leading to cell death (Dickson and Lippman, 2001). On the other hand, mutations of p53 causes genetic instability in the breast cancer cell. This results in the formation of various other mutations probably increasing the survival of the cancer cell.

BRCA1 and BRCA2 genes have a role in the hereditary forms of breast cancer. BRCA1 gene is located on chromosome 17 and does not appear to be a classic tumor suppressor gene of relevance to both tumor onset and progression (Hall JM *et al.*, 1990). BRCA1 mutations

are detected in patients with familial breast and ovarian cancers. On the other hand, BRCA2 gene is located on chromosome 13 (Wooster R *et al.*, 1995). It is generally mutated in both female and male breast cancers in the family. Detection of mutated forms of these two genes are useful to assess the risk of the individual for breast cancer.

Epidermal growth factors also have a stimulatory role on the epithelial cells of the normal breast. In breast cancer, gene amplification and overexpression of c-erb-b2, a member of epidermal growth factor family, is found in approximately 25% of the patients (Hynes and Stern, 1994). Although overexpression of c-erb-b2 is a poor prognostic sign, targeted therapies involving its receptor are given to the patients (Dickson and Lippman, 2001).

Steroid hormone receptors, namely, estrogen and progesterone receptors, are known to influence the prognosis and the response to therapy. Estrogen and progesterone working together modulate the growth, differentiation, and survival of epithelial cells in the breast (Elledge and Fuqua, 2000). Both estrogen and progesterone act through their nucler receptors. The activation of both receptors result in the modulation of transcription of other genes (Dickson and Russo, 2000). In breast cancer, the detection of either estrogen (ER) or progesterone receptor (PR) leads to the application of hormone therapy. As a result, receptor positivity is a good prognostic parameter for the patients (Elledge and Fuqua, 2000).

1.10 Aim of the Study

The advances in the detection and treatment of breast cancer has led to long-term survival for early stage patients. In contrast, delay in diagnosis decreases the survival rate in breast cancer. Although the incidence of breast cancer is lower in our country compared to Western countries, the percentage of advanced disease is higher. Even though the primary treatment is surgery for early stage breast cancer, in advanced cases chemotherapy is chosen as the primary treatment modality. Chemotherapy is used before surgery to decrease the tumor size and the probability of distant spread of the disease. Thus, initial response to chemotherapy is an important determinant of treatment success in this patient group, generally with a poorer prognosis. Clinical resistance to chemotherapy necessitates the change of drugs used and being aware of the possibility of resistance before initiating first line chemotherapy may save time and money which in turn may increase patient's survival.

In this study, multidrug resistance mechanisms related to *mdr1* and *mrp1* genes are evaluated at gene expression and protein levels in locally advanced breast cancer patients (clinically stage IIIB) treated with neoadjuvant chemotherapy. Samples are obtained both before and after chemotherapy. A possible relationship is searched between clinical response and these related genes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

All chemicals used in the experiments were molecular biology grade. Guanidium thiocyanate (GTC), N-laurosylsarcosine, 2-mercaptoethanol, sodium acetate, phenol, ethylenediamine-tetraacetic acid (EDTA), phosphate buffered saline (PBS) tablets, Histopaque-1077, 3-(N-Morpholino) propanesulfonic acid (MOPS), ethidium bromide (EtBr), Tris-HCl, Tris base, glycerol and agarose were purchased from Sigma. Ethanol and xylene were purchased from Riedel de Haen, chloroform was from Lab Scan Analytical Sciences, formaldehyde, sodium citrate, sodium chloride, ammonium acetate, acetic acid were from Merck. Bromophenol blue from Bio-Rad, xylene cyanol from JT-Baker, sodium dodecyl sulfate (SDS) and diethyl pyrocarbonate (DEPC) were from Applichem, M-MuLV-reverse transcriptase (RevertAid^{TM}), Taq DNA polymerase, ribonuclease inhibitor and DNA ladder (GeneRular^{TM} 100 bp DNA Ladder Plus) were from Fermentas. Random hexamer and dNTP were from Promega. RNALater^{TM}, product of Ambion was purchased from Sigma. For immunohistochemistry, monoclonal antibodies for P-glycoprotein and multidrug resistance-associated protein 1 (MRP1), secondary antibodies with biotin-avidine complexes and alkalene phosphatase and chromogen diaminobenzidine (DAB) were purchased from Nova Castra.

2.2 Patients

Breast cancer patients with locally advanced disease were included in this study. Patients with inflammatory breast cancer were excluded. The required information about the patients and the histopathologic properties of the tumors were recorded from the patients' files. All of the patients were primarily treated by chemotherapy. Patients responding to neoadjuvant chemotherapy were operated and modified radical mastectomy was performed in all of these patients.

Patients showing clinical unresponsiveness to the first line drugs either had a second line chemotherapy or radiotherapy in order to control the disease. The responsive patients to these secondary treatment modalities were further treated with surgery. Patients with no response to these treatment methods had surgery only when ulceration or bleeding of the tumor causes morbidity to the patient.

2.3 Cells and Tissues

In this study, surgically removed fresh breast cancer tissues obtained from Turkish patients were used. In order to gain experience and to optimize the RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR), human blood from healthy volunteers and breast cancer patients were used.

2.3.1 Fresh Tissues

Fresh breast cancer tissues were collected from the patients with advanced breast cancer both during initial biopsy performed for the diagnosis of the disease and during surgical removal of the breast, namely mastectomy. Freshly obtained tissues were placed and preserved in RNA Later™ solution which has the ability to protect RNA from degradation. This solution was aliquoted into 1 ml cryogenic vials under aseptic conditions, weighed and stored at +4ºC until further use. The fresh tissues that were approximately 0.5 cm in largest diameter were removed and immediately placed into the solution. The samples were stored at -20ºC until further use for RNA isolation.

2.3.2 Human Blood

Human blood samples from healthy volunteers and breast cancer patients were collected into an EDTA containing tube. After its removal, the blood was utilized in a few hours for RNA isolation in order to prevent RNA degradation.

2.4 Preparation of the Materials for Total RNA Isolation

Both human blood and breast cancer tissues were pretreated before total RNA isolation. This pretreatment procedure differed depending on the material used as stated below.

2.4.1 Pretreatment of Human Blood

Blood was collected into EDTA containing tubes. Onto 3 ml of blood the same volume of cold PBS (Appendix A) was added and mixed vigorously forming a total volume of 6 ml. Into a 15 ml Falcon tube, 6 ml of Histopaque 1077 was added and brought to the room temperature. The blood-PBS mixture was carefully poured onto the Histopaque 1077 without disturbing the phases. The mixture tube was centrifuged at 400 g for 30 min at room temperature. After centrifugation, upper layer was removed and the interphase was collected into a new Falcon tube. The interphase was mixed with 10 ml of PBS and centrifuged at 250 g for 10 min. After discarding the supernatant, 500 µl of GTC solution was added onto the pellet. After dissolving the pellet, the tube content was transferred into a 2 ml Eppendorf tube and the procedure described in section 2.5 was applied.

2.4.2 Pretreatment of Fresh Tissue

The fresh tissue sample was placed in a pre-chilled mortar and liquid nitrogen was poured immediately onto the tissue covering it completely. Then the tissue was grinded rigorously with a pestle until it became powder. The grinded tissue was covered with 2 ml of GTC solution and transferred into a 15 ml Falcon centrifuge tube and vortexed vigorously. Aliquots of 500 µl were transferred into 2 ml Eppendorf tubes and three of them were stored at -80°C for further use and the fourth one was used for RNA isolation according to the procedure described in section 2.5.

2.5 Total RNA Isolation

The method for RNA isolation from the blood and the tissues was a modification of the protocol offered by Chomczynski and Sacchi (1987) (Gauthier ER et al., 1997). In order to decrease the possibility of RNA degradation during the procedure, all glassware and plasticware were treated by incubating them overnight in 0.01% DEPC. After incubation, all of the material used for isolation were autoclaved and dried in the oven. All of the solutions were either DEPC treated or prepared in DEPC treated water.

After the preparation phase, appropriate amount of GTC solution was added onto both the blood and the tissue samples. Then, the following solutions were added in volumes relative to the volume of GTC solution. In sequence, 2.0 M sodium acetate (pH 4.0) in 1/10 volume, 0.1 M citrate buffer (pH 4.3) saturated phenol in 1/1 volume and cholorofom in 1/5 volume of GTC were added onto the GTC-tissue or GTC-blood mixture and vortexed after addition of each solution. After these steps, the samples were incubated for 15 min on ice and centrifuged at 13000 rpm for 20 min (MSE Benchtop Microcentrifuge). After centrifugation, the upper aqueous phase was collected into a fresh 1.5 ml Eppendorf tube (maximum 500 µl). Onto the aqueous phase, two volumes of ice cold 96% ethanol was added (maximum 1000 µl) and incubated for 20 min at -20°C. After the incubation, the tubes were centrifuged at 13000 rpm for 10 min, the supernatant was discarded and 400 µl of 70% ethanol was added onto the pellet. After a second similar centrifugation, the supernatant was carefully pipetted. The remaining ethanol was removed by drying in the oven (55-60°C). Onto the pellet 30 µl of sterile pyrogen free water was added and incubated for 10 min at 60-65°C. The isolated RNA was used in the subsequent steps. The remaining RNA was stored after rapid freezing in liquid nitrogen at -80°C for further use if necessary.

2.5.1 Spectrophotometric Analysis of RNA

For this procedure, 10 µl of total RNA isolate was diluted with 990 µl of TE buffer (pH 8.0) (Appendix A) in a quartz cuvet. Then, the absorbance of the solution was measured at 260 and 280 nm wavelenghts by a Shimadzu UV-1208 spectrophotometer using TE buffer as blank. The purity of the isolated RNA was determined by taking the ratio of A_{260} and A_{280} readings. The optimal value for RNA purity is accepted to be between 1.9-2.2 (MacDonald RJ *et al.*, 1987).

2.5.2 Agarose Gel Electrophoresis of Total RNA

Agarose gel electrophoresis was used for the visualization of the intactness of isolated RNA. The electrophoresis was performed under denaturating or non-denaturating conditions.

2.5.2.1 Denaturating Agarose Gel Electrophoresis

One percent of agarose gel was prepared in 1X MOPS buffer (pH 7.0) (Appendix A) in a total volume of 25 ml. Formaldehyde was added in order to have denaturating conditions (final concentration was 6%) (Kitlinska and Wojcierowski, 1995). Ten µl of total RNA was mixed with 1 µl of loading solution (Appendix A) and loaded onto the gel. After running for an hour at 70V (Apelex PS-503 power supply), the gel was stained in 0.5 mg/ml EtBr (Appendix A) solution containing 0.1 M ammonium acetate (Kitlinska and Wojcierowski, 1995) at least for an hour. The RNA bands were visualized under UV transilluminator and photographed (Vilber Lourmat Gel Imaging and Analysis System).

2.5.2.2 Non-Denaturating Agarose Gel Electrophoresis

In this procedure, 10 µl of total RNA isolate was mixed with 1 µl of loading solution (Appendix A) and loaded onto a 1% agarose prepared in 25 ml 1X TAE buffer (Appendix A). The gel was run at 70V for an hour. The gel was stained in 0.5 mg/ml EtBr solution for about 10 min. Then the gel was visualized under UV light and a photograph was taken.

2.6 Complementary DNA (cDNA) Preparation

After RNA isolation, RNA was immediately reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV-RT). For the reaction, 1-2 µg of RNA was used corresponding to 1-10 µl of total RNA isolate.

2.6.1 cDNA Preparation from Fresh Tissue Samples

RNA isolated from fresh tissue samples was reverse transcribed where oligo-dT was used as a primer. Into a 0.6 ml Eppendorf PCR tube,1-2 µg of RNA (at least 1 µl at most 10 µl), 2 µl of oligo-dT (stock was 0.5 µg/µl) was added and the total volume was completed up to 12 µl with pyrogen free sterile water. The tube was incubated at 70°C for 5 min (COY

Themcycler) and chilled on ice. Then, 4 µl of 5X M-MuLV-reverse transcriptase buffer (final concentration 1X), 2 µl of 10 mM dNTP mix (final concentration 1.0 mM), and 1 µl of RNase inhibitor (20 U/µl) were added in the indicated order. After incubating at 37°C for 5 min, 1 µl M-MuLV-reverse transcriptase (200 U/µl) was added. The reaction was carried out at 42°C for 60 min. Finally, the tube was heated up to 70°C for 10 min and chilled on ice. The samples were stored at -20°C until further use.

2.7 The Polymerase Chain Reaction (PCR)

The cDNA obtained was amplified by PCR. Multiplex PCR was used to amplify *mdr1* and *mrp1* genes. In multiplex PCR, each time more than one gene is amplified and either *mdr1* or *mrp1* gene is amplified with β_2M gene in this study.

2.7.1 Primers Used During PCR

In order to amplify a gene by PCR, primers were designed for a specific part of the gene. In this study, specific primers for both *mdr1* and *mrp1* genes were designed. A constitutively expressed gene, namely beta-2-microglobulin (β_2M), was chosen in order to assess the quality of PCR.

Mrp1 primers (Ferrero JM *et al.*, 2000), *mdr1* primers (Dexter DW *et al.*, 1998) and β_2M primers (Dexter DW *et al.*, 1998) were prepared by IDT Technologies, USA. The sequence of the primers used to amplify *mdr1*, *mrp1* and β_2M genes are shown in Table 2.1. The final PCR product was 167 bp for *mdr1* gene, 291 bp for *mrp1* gene and 115 bp for β_2M gene.

Table 2.1 Sequence of the primers used in the study

Name of the Primer	Sequence of the Primer (5'-3')
mdr1 forward	5'-cccatcattgcaatagcagg
mdr1 reverse	5'-gttcaaacttctgctcctga
mrp1 forward	5'-ggacctggacttcgttctca
mrp1 reverse	5'-cgtccagacttcttcatccg
β_2M forward	5'-accccactgaaaaagatga
β_2M reverse	5'-atcttcaaacctccatgatg

2.7.2 The Multiplex PCR

Multiplex PCR reaction was performed by the simultaneous amplification of the different genes in the same reaction tube. The reaction was performed by simply adding the following items in the indicated order into a 1.5 ml Eppendorf tube to produce a master mix:

	Stock Solution	Final Concentration
Pyrogen free sterile H_2O	-	up to 50 µl
Reaction buffer (App A)	10X	1X
$MgCl_2$	25 mM	1.5 mM
dNTP mix	10 mM (each)	0.1 mM (each)
mrp1 forward / reverse primer	100 µM (each)	1.0 µM (each)
mdr1 forward /reverse primer	100 µM (each)	1.0 µM (each)
β_2M forward / reverse primer	250 µM (each)	0.3 µM (each)

Either *mrp1* or *mdr1* primers were used.

After the master mix was prepared, it was aliquoted (47.5 µl) into fresh 0.6 ml Eppendorf PCR tubes. Then, 2 µl from the cDNA reaction mixture (no more than 1/10 of volume) and 0.5 µl Taq DNA polymerase (final concentration 2.5 U/µl) were added making a final volume of 50 µl.

The thermal cycler (Techne Techgene, England) was programmed as;

Initial denaturation at 94°C for 3 min

Denaturation at 94°C for 30 sec

Annealing at 60°C for 30 sec

Extension at 72°C for 45 sec

These steps were repeated for 30 cycles.

Final extension at 72°C for 5 min

Final hold at 4°C indefinitely

After the reaction was completed, PCR tubes were stored at -20°C until further use.

2.8 Agarose Gel Electrophoresis of PCR Products

In a total volume of 25 ml, 2% agarose and 1X TBE buffer (Appendix A) were prepared and poured onto a gel tray. To the first well, 0.25 µg/µl of DNA Ladder (100 bp-3000 bp) was loaded. Seven µl of each PCR product were mixed with the loading dye (Appendix A). The mixture was loaded to each well. The gel was run at 90 V for 60 min and was stained in a staining solution containing 0.5 mg/ml EtBr for 5-10 min. The gel was then visualized under ultraviolet transilluminator and the photographed.

2.9 Densitometric Analysis of PCR Products

In order to quantify the PCR products on agarose gels, densitometric measurements were done. The photographs of the multiplex PCR gels were analyzed using Scion Image Software (Scion Corporation, USA). The intensities of the bands were converted into peaks by the software. The *mdr1, mrp1* and $\beta_2 M$ gene expression products were calculated from the area under these peaks. In order to obtain a measured value for each patient having gene expression, the intensity values of the bands of *mdr1* and *mrp1* genes were divided by the intensity values of the bands of $\beta_2 M$ gene in the same lane on the agarose gel. Thus, a ratio, as shown below, was obtained for each patient both before and after chemotherapy.

$$\text{Ratio (P)} = \frac{\text{Densitometric intensity of } mdr1/mrp1 \text{ band of the patient}}{\text{Densitometric intensity of } \beta_2 M \text{ band of the patient}}$$

The ratio values obtained before and after chemotherapy for each patient were compared to detect a difference in gene expression. This comparison is especially useful for *mrp1* gene expression which was abundantly present both before and after chemotherapy.

2.10 Detection of the Resistance Proteins by Monoclonal Antibody Staining

Immunohistochemistry (IHC) was used to demonstrate the presence of protein products of *mdr1* and *mrp1* genes, namely P-glycoprotein (Pgp) and MRP1, in breast cancer tissues. For this purpose, monoclonal antibodies, JSB-1 and QCRL-1, were used for Pgp and MRP1 proteins respectively. JSB-1 antibody is an Ig G1 type of antibody and it recognizes an

internal cytoplasmic epitope. The antibodies used for the detection of p53 (DO-7 + BP53-12), c-erb-b2 (e2-4001 + 3B5), ER (1D5 + 6F11) and PR (hPRa2 + hPRa3) were purchased from Neomarkers, USA..

In this method, proteins localized either on the cell membrane or inside the cell were stained using monoclonal antibodies recognizing their antigens. Secondary antibodies specific for the primary ones and containing biotin on the other end of the molecule were applied. Then, peroxidase system containing avidine, a molecule capable of binding biotin, on one end and peroxidase enzyme on the other was bound to the secondary antibodies. Finally, a chromogen, diaminobenzidine (DAB), which shows a color change due to peroxidase activity, was added. The final color for detected resistance proteins was brown.

2.10.1 Staining Procedure

Initially, 4 μ thick sections from formalin fixed, paraffinized tissues were put on positively charged slides. Positively charged slides were used to increase the stability of the tissues on the slides. Tissue sections were deparaffinized with overnight incubation at 37°C and this procedure increased the attachment of the samples to the slides. Deparaffinization was completed by washing the slides in two different xylol solutions for 5 min each. Tissue sections were hydrated twice in ethyl alcohol solutions with 96% concentration for 3 min and finally placed into distilled water.

Following this, slides were placed into 3% hydrogen peroxide solution for 20 min in order to inactivate the endogenous peroxidase activity. Slides were then washed in 0.5 M Tris buffer (pH=7.6) for 10 min. In order to break the bonds formed by formalin and regain the antigens on the cell surface, slides were placed in 0.01 M trisodium citrate (pH=6) solution and boiled in microwave oven at maximum power (650 W) twice for 8 min and between the two boilings a 10 min interval was given. During and between the two boilings the decreased amount of citrate was replenished. After cooling for 20 min, slides were placed in Tris buffer to prevent drying.

After this, tissues on the slides were marked circumferentially with a hydrophobic isolation pen to prevent the dissemination of the solutions added in the following steps. Then, protein block containing milk was added. After 5 min, excess amount of protein block was rinsed and primary monoclonal antibodies were added. Tissue sections were incubated at room temperature for 2 hours and then washed in Tris buffer for 20 min. Biotinylated secondary

antibodies were added and after 10 min incubation, slides were washed in Tris buffer. Then streptavidine-peroxidase complex was added and after another 10 min incubation, slides were washed in Tris buffer once again. The chromogen, diaminobenzidine (DAB) solution, was added and after 20 min incubation, slides were washed in distilled water. Finally, slides were counterstained with Mayer's hematoxylene stain for 5 min and dried and covered with balsam ready for examining under light microscopy.

2.10.2 Evaluation of the Staining and Quantification of the Resistance

The staining patterns of the tissues were evaluated by counting the number of positively stained cells in four different representative areas of the slide under 40 x magnification and the average count was calculated. Since Pgp generally showed less intense staining pattern, this method was primarily used to calculate the staining intensity for MRP1. According to the number of stained cells, the patients had 0-100% positivity. The percentage of positivity was compared between pre- and post-chemotherapy samples of the patients.

CHAPTER 3

RESULTS

3.1 The Patients

In this study, twenty-five patients with locally advanced breast cancer were included. All patients had T4 tumors invading either the breast skin or underlying muscles detected by physical examination and mammography and were clinically in stage IIIB. Patients with inflammatory breast cancer were excluded from the study. All patients were female with a median age of 53 (range 34-75).

Fresh tissue samples were obtained from the patients both during initial biopsy and following mastectomy. All of these patients had both pre- and post-chemotherapy tissue samples. Clinicopathologic properties of the patients are depicted in Table 3.1. Patients responding to chemotherapy received 3-5 courses of cyclophosphamide (600 mg/m^2), epirubicin or doxorubicin (60 mg/m^2) and 5-fluorouracil (600 mg/m^2) combination repeated in every 21 days. Clinical response was usually evaluated after 3-4 courses and either up to 5 courses were given or the chemotherapy drugs were changed in clinically non-responders. In addition, radiotherapy was given to further increase the clinical response.

Clinical response to chemotherapy was accepted as a decrease in either the tumor size or skin edema or a decrease in either the number or fixation of clinically detected axillary lymph nodes. During chemotherapy, clinical unresponsiveness developed in five patients (20%) (P 4, 5, 13, 22, 23). Chemotherapy drugs were changed in two of these patients. One of them (P 4) received vinorelbine and mitoxantrone after 8 courses of cyclophophamide, doxorubicin

Table 3.1. Clinicopathologic properties of the patients

Patient	Tumor size (cm)		Lymph node status		Chemotherapy
	Pre-CT	Post-CT	Pre-CT	Post-Surgery*	
P 1	4x4	3x1.5x1.5	+	13/15	Cyclo/Epi/5-FU 4 cycles
P 2	5x4	3x2.5x1.5	--	6/13	Cyclo/Epi/5-FU 4 cycles
P 3	9x8	5x2x2	+	20/30	Cyclo/Epi/5-FU 4 cycles
P 4	6x5	8x5x3.5	+		Cyclo/Doxo/5-FU 8 cycles Vino/Mitox 8 cycles
P 5	10x8	12x9x3.5	+	26/27	Cyclo/Epi/5-FU 5 cycles + RT
P 6	8x7	4x3x1.5	+	17/32	Cyclo/Epi/5-FU 3 cycles
P 7	8x7	2x1.5x1	+	3/12	Cyclo/Epi/5-FU 4 cycles
P 8	7x6	5x4x3.5	+	8/8	Cyclo/Epi/5-FU 3 cycles
P 9	6x5	6x5.5x2	+	12/12	Cyclo/Epi/5-FU 4 cycles
P 10	10x8	6x5x4	+	0/25	Cyclo/Epi/5-FU 5 cycles
P 11	8x5	2.5x2x2	+	4/34	Cyclo/Epi/5-FU 3 cycles
P 12	11x10	5x4x4	+	11/27	Cyclo/Epi/5-FU 3 cycles
P 13	6x3	5x3x2	+	8/8	Cyclo/Epi/5-FU 4 cycles Docetaksel 2 cycles
P 14	7x5	4x3.5x2	+	4/21	Cyclo/Epi/5-FU 3 cycles
P 15	12x10	6x5x3	+	5/24	Cyclo/Epi/5-FU 3 cycles
P 16	7x7	5x3x3	+	2/26	Cyclo/Epi/5-FU 3 cycles
P 17	6x5	3x3x2	+	14/28	Cyclo/Epi/5-FU 3 cycles
P 18	5x4	2x2x1	+	12/18	Cyclo/Doxo/5-FU 4 cycles
P 19	5x5	3x2x2	+	4/19	Cyclo/Epi/5-FU 3 cycles
P 20	8x7	5x5x4	+	0/22	Cyclo/Epi/5-FU 3 cycles
P 21	12X10	4.5x3.5x2	+	8/24	Cyclo/Doxo/5-FU 3 cycles
P 22	6X5	6X5X4	+	5/14	Cyclo/Epi/5-FU 5 cycles
P 23	7x5	7x4x2	+	10/11	Cyclo/Doxo/5-FU 4 cycles + RT
P 24	6x6	4x3x2	+	11/14	Cyclo/Doxo/5-FU 4 cycles
P 25	11x10	4x4x3	+	6/17	Cyclo/Epi/5-FU 3 cycles

***** Number of metastatic lymph nodes divided by total number of dissected lymph nodes

and 5-fluorouracil therapy and the other one (P 13) received docetaxel after 4 courses of cyclophophamide, epirubicin and 5-fluorouracil. Surgery was performed when a response to new drugs was obtained after two courses in P13. In contrast, although P4 did not respond to the change in drugs, surgery was performed for ulceration and bleeding in the diseased breast. In other two clinically unresponsive patients (P 5, 23), preoperative radiotherapy was applied additionally. A response to radiotherapy was detected and these patients were further treated with surgery. The fifth patient (P 22) had no response to drugs and surgery was performed due to ulceration and bleeding in the diseased breast despite the clinical unresponsiveness.

3.2 Total RNA Isolation and Optimization of Methods Using Human Blood Samples

Total RNA isolation was initially performed from peripheral blood samples of healthy volunteers and breast cancer patients to optimize the isolation procedure. Total RNA was isolated and the purity of RNA was evaluated by spectrophotometric analysis. OD_{260}/OD_{280} values for samples obtained changed between 1.7 to 2.0. This range indicated an acceptable purity for isolated RNA. After RNA isolation, cDNAs were obtained by converting RNA with reverse transcription. Then, these cDNAs were amplified by PCR using specific primers. PCR products were electrophoresed on agarose gel and the bands were visualized using ethidium bromide.

Both *mdr1* and *mrp1* genes were found to be rarely expressed in blood samples of healthy volunteers. In contrast, breast cancer patients had frequent expression of these genes compared to healthy subjects. Optimization of the isolation method and RT-PCR was performed using the blood samples. After this, total RNA isolation from breast tissues of 25 patients was performed.

3.3 Expression of *mdr1* Gene and Clinical Response to Chemotherapy

The results of *mdr1* gene expression both before and after chemotherapy and its relation to clinical response to chemotherapy are given in Table 3.2. Before chemotherapy, *mdr1* gene expression was detected in 3 patients (12%) (P 1, 2, 4). Two of the initially *mdr1* gene expressing patients (P1, 4) had persistent *mdr1* expression after chemotherapy and P4 was

clinically unresponsive in accordance with these results. In contrast, P1 expressed *mdr1* gene initially and even though the expression continued during chemotherapy, a partial clinical response was obtained. Besides, *mdr1* gene expression was not detected in P2 after chemotherapy and the patient had clinical response.

After chemotherapy, *mdr1* gene expression started in 4 patients (16%) (P 5, 13, 22, 23) and all of these patients (100%) were in the clinically unresponsive patient group (Fig.3.1). Overall, *mdr1* gene expression was found to be related to clinical drug response.

3.4 Expression of *mrp1* Gene and Clinical Response to Chemotherapy

The results of *mrp1* gene expression and its relation to clinical response are shown in Table 3.2. In contrast to *mdr1* gene, *mrp1* gene was found to be expressed abundantly before chemotherapy. In 68% (17/25) of the patients, *mrp1* gene expression was detected. All of these initially *mrp1* expressing patients had continous expression after chemotherapy. The *mrp1* gene expression results of selected patients are shown in Fig. 3.2.

In addition, in four more patients, *mrp1* gene expression was detected after chemotherapy. As a total, 84% (21/25) of the patients had *mrp1* gene expression at the end of the chemotherapy. In this group, 80% (4/5) of the clinically unresponsive patients (P 4, 5, 13, 23) showed *mrp1* gene expression. Among these, three patients (P 4, 5, 23) had *mrp1* expression both before and after chemotherapy where as in P 13, *mrp1* expression started after chemotherapy. P 22 had no *mrp1* gene expression either before or after chemotherapy although a clinical unresponsiveness was detected. As a result, expression rate of *mrp1* gene was higher in clinically responsive patients compared to clinically unresponsive patients (85% vs 80%).

3.5 The Results of Densitometric Analysis of PCR Products

Densitometric analysis was performed for the bands obtained upon gel electrophoresis of RT-PCR products as described in Section 2.9. The results are given in Table 3.3 and 3.4. For *mdr1*, the ratios of band intensities of *mdr1* to $\beta_2 M$ were around one in all patients where bands were observed on gels (Table 3.3). Although *mdr1* gene expression was observed for P 1 both before and after chemotherapy (0.92 and 0.94, expression levels respectively),

Table 3.2. Results of gene expression, protein detection and clinical response of patients

Patient	Pre-chemotherapy				Post-chemotherapy				*** Clinical Response
	Protein Detection		Gene Expression		Protein Detection		Gene Expression		
	P-gp*	MRP**	*mdr1*	mrp1	P-gp	MRP	*mdr1*	mrp1	
P 1	+	_	+	_	+	_	+	_	+
P 2	+, cyt	60%	+	+	_	70%	_	+	+
P 3	_	70%	_	+	+	95%	_	+	+
P 4	_	30%	+	+	+	90%	+	+	_
P 5	_	30%	_	+	+	100%	+	+	_
P 6	_	10%	_	+	_	20%	_	+	+
P 7	_	10%	_	+	_	80%	_	+	+
P 8	_	70%	_	+	_	100%	_	+	+
P 9	_	60%	_	+	_	100%	_	+	+
P 10	_	_	_	_	_	_	_	_	+
P 11	+, cyt	30%	_	+	_	100%	_	+	+
P 12	_	50%	_	+	_	40%	_	+	+
P 13	_	_	_	_	+	40%	+	+	_
P 14	_	_	_	_	_	30%	_	+	+
P 15	_	_	_	_	_	_	_	_	+
P 16	_	10%	_	+	+, cyt	90%	_	+	+
P 17	_	70%	_	+	_	100%	_	+	+
P 18	+, rare	100%	_	+	+, rare	100%	_	+	+
P 19	_	100%	_	+	_	100%	_	+	+
P 20	_	100%	_	+	_	100%	_	+	+
P 21	_	100%	_	+	_	100%	_	+	+
P 22	_	_	_	_	+	_	+	_	_
P 23	_	10%	_	+	+	90%	+	+	_
P 24	_	_	_	_	_	80%	_	+	+
P 25	_	_	_	_	_	10%	_	+	+

* cyt: cytoplasmic staining (otherwise membranous); rare: rare membranous staining
** MRP1 levels are assigned by counting the antibody stained cells under microscope (Fig. 3.4)
*** (+): clinically responsive patients; (-) clinically unresponsive patients

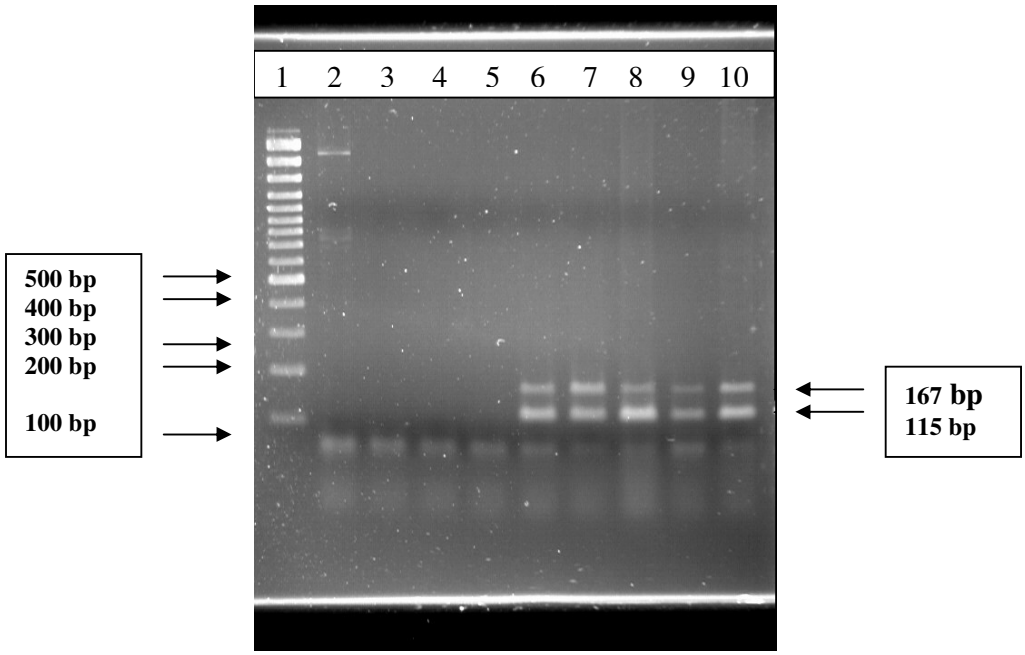


Fig. 3.1. RT-PCR results for *mdr1* gene. Lane 1 – DNA ladder. Lanes 2-5 RT-PCR products of indicating *mdr1* gene expression before chemotherapy for P 5, P 13, P 22, and P 23, respectively. Lanes 6-10 show RT-PCR products indicating *mdr1* gene expression after chemotherapy for P 4, P 5, P 13, P 22, and P 23, respectively.

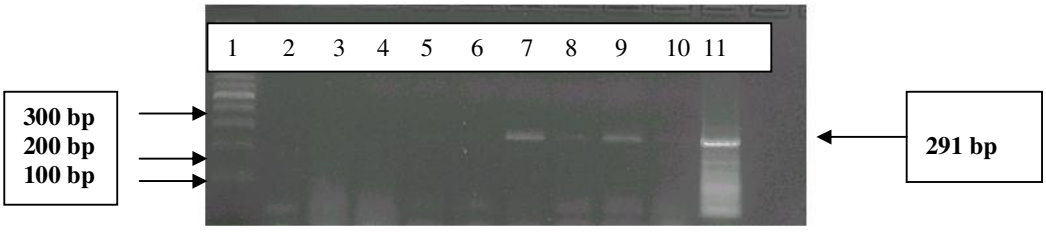


Fig. 3.2. RT-PCR results for *mrp1* gene. Lanes 1 and 11 – DNA ladder. Lanes 2-6 show the expression of *mrp1* before chemotherapy for patients P1, P10, P13, P 14 and P 22, respectively. Lanes 7-10 showing results after chemotherapy for patients P 4, P 5, P 13 and P 22, respectively.

clinical unresponsiveness was not observed in this patient. While P 2 had a weak band for *mdr1* gene product (0.84, expression level) before chemotherapy, it was not detected after chemotherapy. This patient clinically responded to chemotherapy.

Except these two cases, an increase in the value of *mdr1/β_2M* ratios was observed after chemotherapy in all other clinically unresponsive patients. Especially for P 5, P 13, P 22 and P 23, the expressions changed very significantly from no expression initially to high expression.

Table 3.3 Results of densitometric analysis for *mdr1* gene expressing patients

	Before Chemotherapy			After Chemotherapy		
Patient	*Mdr1*	*β_2M*	*Mdr1/β_2M*	*Mdr1*	*β_2M*	*Mdr1/β_2M*
P 1	1180	1283	0.92	1273	1354	0.94
P 2	1217	1449	0.84	--	--	--
P 4	1326	1044	1.27	1386	969	1.43
P 5	--	--	--	1196	712	1.68
P 13	--	--	--	1157	918	1.26
P 22	--	--	--	1147	760	1.51
P 23	--	--	--	1446	836	1.73

β_2M; beta-2-microglobulin

In case of *mrp1* expression, increasing *mrp1/β_2M* ratios were observed after chemotherapy in almost all patients where there is any expression. The mean value of the expression levels before chemotherapy was 1.17 whereas this value increased to 1.26 after chemotherapy. The mean values obtained before and after chemotherapy were compared using Student's t test. Although a tendecy for an increase in *mrp1* expression was found after chemoterapy, this difference did not reach statistical significance (p=0.07). The increase in band intensities which indicate increased gene expression is in accordance with the increase observed in the percentage of staining cells with QCRL-1 antibodies as discussed in Section 3.7.

In clinically unresponsive patients (P 4, P 5, P 13, P 22, P 23), the ratio is increasing after chemotherapy except in P 22 where there is no expression both before and after chemotherapy. The increase in P 13 is quite significant.

Table 3.4 Results of densitometric analysis for *mrp1* gene

	Before Chemotherapy			After Chemotherapy		
Patient	*Mrp1*	β_2M	*Mrp1*/β_2M	*Mrp1*	β_2M	*Mrp1*/β_2M
P 1	--	--	--	--	--	--
P 2	1317	1208	1.09	1254	1063	1.18
P 3	1128	918	1.23	1045	810	1.29
P 4	1338	1143	1.17	1426	1072	1.33
P 5	1078	1123	0.96	1274	995	1.28
P 6	1264	1118	1.13	1253	1053	1.19
P 7	1332	1233	1.08	1554	1273	1.22
P 8	1196	1058	1.13	1204	979	1.23
P 9	1225	1038	1.18	1229	1016	1.21
P 10	--	--	--	--	--	--
P 11	1298	1138	1.14	1607	1173	1.37
P 12	1006	845	1.19	1244	1037	1.20
P 13	--	--	--	1153	901	1.28
P 14	--	--	--	1128	981	1.15
P 15	--	--	--	--	--	--
P 16	1110	917	1.21	1254	980	1.28
P 17	1214	1003	1.21	1196	997	1.20
P 18	1320	963	1.37	1342	965	1.39
P 19	1417	1107	1.28	1356	1035	1.31
P 20	1042	876	1.19	1006	818	1.23
P 21	1232	1010	1.22	1312	1017	1.29
P 22	--	--	--	--	--	--
P 23	1244	1101	1.13	1558	1097	1.42
P 24	--	--	--	1112	800	1.39
P 25	--	--	--	1315	1164	1.13

β_2M*;* beta-2-microglobulin

3.6 Optimization of Immunohistochemistry Staining

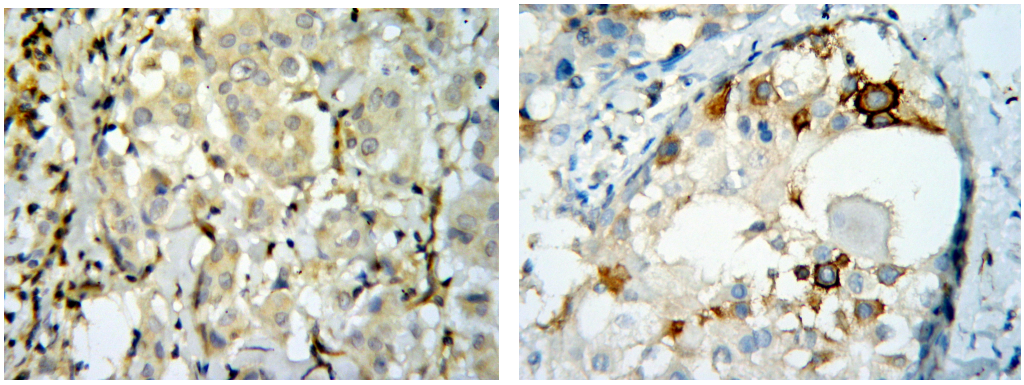
The concentration of the monoclonal antibodies was optimized to obtain the best staining results. In order to optimize the immunohistochemistry staining, initially normal breast and kidney tissues were stained. Normal breast tissue did not show a strong staining pattern both for Pgp and MRP1. In contrast, kidney tissue stained strongly with Pgp and MRP1. As a result, kidney tissue was chosen, since it strongly expresses these proteins and it was

accepted as the positive control for further studies. On these tissues, the optimal staining dilution both for JSB-1 and QCRL-1 was determined. Both of these monoclonal antibodies were diluted to 1/20, 1/40, 1/80 and 1/160 concentrations. Tissues were stained using these diluted antibodies. The optimal staining was obtained between 1/20 – 1/40 dilutions. In sample tissues, lymphocytes were stained in addition and used as internal controls in breast tissue samples as these cells are known to contain Pgp and MRP1 abundantly. In the normal breast tissue, only ductal epithelium showed a weak staining pattern as expected. In the breast cancer tissues, the staining was found optimal at 1/20 dilution for JSB-1 and at 1/40 dilution for QCRL-1. Breast cancer cells showed both membranous and cytoplasmic staining with JSB-1 and QCRL-1 antibodies in different patients. Only the membranous staining was considered as true positive staining.

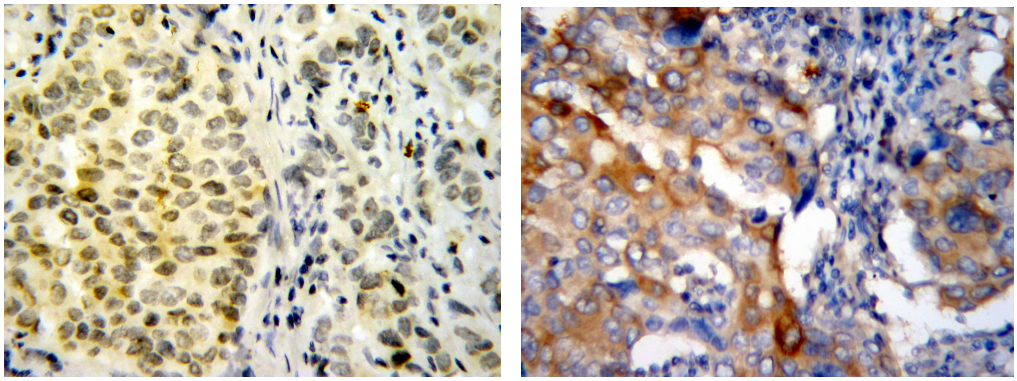
3.7 Immunohistochemistry Staining for P-glycoprotein

The staining results of the patients for Pgp are shown in Table 3.2. In addition, representative pictures of a patient (P 3) showing Pgp positivity after chemotherapy is given in Fig. 3.3. By immunohistochemical staining, four of the patients (P 1, 2, 11, 18) were Pgp positive before chemotherapy. Two of the initially Pgp positive patients (P 2, 11) did not show Pgp staining after chemotherapy and clinically these patients responded to chemotherapy. In contrast to the usual membranous staining pattern, the staining pattern was cytoplasmic in these patients. P 11 had no detectable *mdr1* gene expression both before and after chemotherapy where as the expression of *mdr1* gene was detected before chemotherapy and disappeared after chemotherapy in P 2. In addition, Pgp staining was rare in tissue samples of P 18 both before and after chemotherapy. Besides, *mdr1* expression was not detected in this patient as well. In contrast, P1 had persistant Pgp positivity after chemotherapy and despite this a partial response to chemotherapy was obtained. This result was similar to RT-PCR results of this patient in that *mdr1* gene expression was detected both before and after chemotherapy. Densitometric analysis revealed that the expression ratio of $mdr1/\beta_2M$ was relatively low and was not affected by chemotherapy (Table 3.3).

On the other hand, Pgp positivity was induced in 7 patients (28%) (P 3, 4, 5, 13, 16, 22, 23) after chemotherapy, including all of the five clinically unresponsive patients. Among these patients, P 16 showed only cytoplasmic granular staining pattern. Although P 3 and P 16 had positive staining with IHC, *mdr1* gene expression was not detected by RT-PCR in these patients both before and after chemotherapy and they clinically responded to chemotherapy.

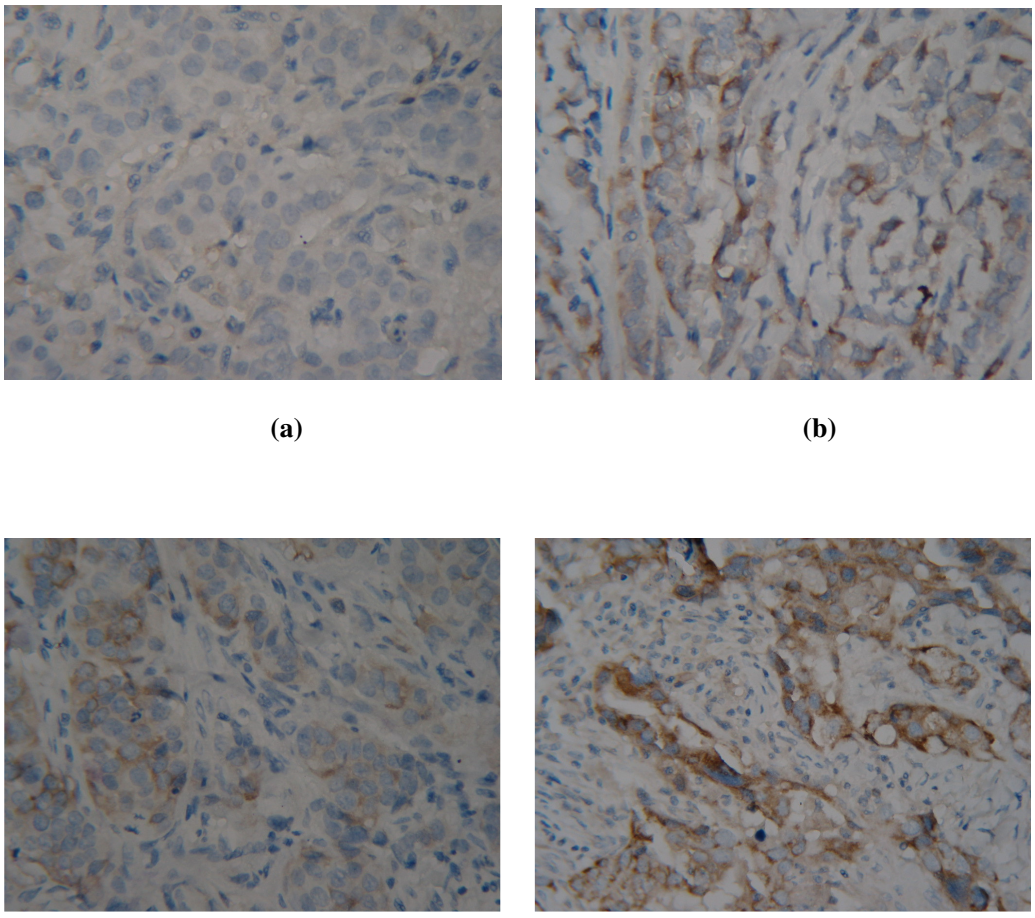


(a) **(b)**



(c) **(d)**

Fig. 3.3. The results for protein products of *mdr1* and *mrp1* genes by immunohistochemistry. (a) Pgp negative result for P3 before chemotherapy. (b) Pgp positivity detected after chemotherapy for P3. (c) MRP1 negativity of P14 before chemotherapy. (d) MRP1 positivity of P14 after chemotherapy.



(a) **(b)**

(c) **(d)**

Fig. 3.4. Quantification of MRP1 antibody staining in breast cancer tissues.
(a) Negative staining (b) 10% staining (c) 50% staining (d) 100% staining

Therefore, Pgp staining observed in these two may be due to false positivity of IHC procedure since there were no detectable *mdr1* gene expression.

3.8 Immunohistochemistry Staining for MRP1

Selected pictures of breast cancer tissue of a patient with induced MRP1 positivity are shown in Fig. 3.3. Similar to *mrp1* gene expression, MRP1 positivity was found in 17 patients (68%) with IHC before chemotherapy. The percentage of staining cells changed between 10-100% in these patients and is shown in Table 3.2. The representative figures indicating no staining, 10%, 50%, and 100% staining patterns are included in Fig. 3.4. In addition, the number of MRP1 stained cells and counted cells and their ratios for all of the patients both before and after chemotherapy are given in Appendix B.

MRP1 positivity increased after chemotherapy in parallel to *mrp1* expression. Thus, 21 patients (84%) showed MRP1 positivity after chemotherapy. In four of the five clinically unresponsive patients, the tissues stained positive for MRP1. The percentage of the stained cells generally increased after chemotherapy in clinically unresponsive patients implying an increased activity of MRP1 protein.

3.9 Statistical Analysis

The relationship between *mdr1* and *mrp1* gene expressions and clinical response was evaluated statistically using Fisher's exact test. The results are shown in Table 3.5. *mdr1* gene expression detected after chemotherapy and clinical response to chemotherapy were significantly related in locally advanced breast cancer patients ($p<0.001$). Similarly, patients showing Pgp staining after chemotherapy were clinically more unresponsive and the difference was significant ($p<0.001$). Although there is a definite increase in the degree of *mrp1* gene expression after chemotherapy, this increase does not seem to be statistically significant.

3.10 Correlation of Clinicopathologic Properties of Tumor and MDR Genes

The clinicopathologic properties of the patients are shown in Table 3.1 and Table 3.6. Estrogen (ER) and progesteron receptor (PR), p53 and c-erb-b2 proteins were detected with IHC in these patients as a part of the routine diagnostic work-up. The number of patients showing ER and PR positivity was 18 (72%) and 21 (84%), respectively. Mutant p53 positivity was detected in 16 patients (64%). On the other hand, c-erb-b2 positivity was present in 11 patients (44%). The distribution of *mdr1* and *mrp1* gene expression and ER, PR, p53 and c-erb-b2 status was statistically evaluated using Fisher's exact test. The distribution of *mdr1* or *mrp1* gene expressing patients in ER, PR, p53 and c-erb-b2 positive groups both before and after chemotherapy was not significant.

Table 3.5. The distribution of multidrug resistance gene expressing patients in relation to clinical response after chemotherapy

	Clinical Response		**Statistical Analysis**
	Drug Unresponsive	**Drug Responsive**	**p value**
***Mdr1* gene expression ***			
no induction	**1**	**20**	
induction	**4**	**_**	**< 0.001**
Pgp **			
negative	**_**	**18**	
positive	**5**	**2**	**< 0.001**
***Mrp1* gene expression ***			
no induction	**4**	**17**	
induction	**1**	**3**	**NS**
MRP1 **			
negative	**4**	**17**	
positive	**1**	**3**	**NS**

* determined by RT-PCR; ** determined by antibody staining; NS: not significant

Table 3.6. Receptor status of the tumors (ER & PR), the expressions of p53 and c-erb-b2 genes in relation to drug resistance profile of the patients

Patient	Pgp	MRP1	*Mdr1*	Mrp1	ER	PR	p53	c-erb-b2
P 1	+	_	+	_	+, weak	_	_	+
P 2	_	70%	_	+	+, str	+, med	+	+, med
P 3	+	95%	_	+	+, med	+, weak	_	_
P 4	+	90%	+	+	+, weak	+, weak	+, str	+
P 5	+	100%	+	+	_	+, str	+	+
P 6	_	20%	_	+	+	+, med	+	_
P 7	_	80%	_	+	+, weak	+, weak	+	_
P 8	_	100%	_	+	+, str	+, str	+	_
P 9	_	100%	_	+	+, str	+, weak	_	_
P 10	_	_	_	_	+, str	+, med	_	+
P 11	_	100%	_	+	+, str	+, str	+	_
P 12	_	40%	_	+	+, str	+, med	+	_
P 13	+	40%	+	+	+, med	+, weak	_	_
P 14	_	30%	_	+	+, str	+, med	_	_
P 15	_	_	_	_	_	+, med	+	+
P 16	+, cyt	90%	_	+	_	+	+	_
P 17	_	100%	_	+	_	_	_	+, str
P 18	+, rare	100%	_	+	+, med	_	_	+, str
P 19	_	100%	_	+	+, med	+, med	_	+
P 20	_	100%	_	+	_	+	+	+
P 21	_	100%	_	+	+, med	+, str	+	+, str
P 22	+	_	+	_	_	+, med	+	_
P 23	+	90%	+	+	+, str	+, med	+	_
P 24	_	80%	_	+	+, med	+, str	+, med	_
P 25	_	10%	_	+	_	_	+	_

ER: estrogen receptor; PR: progesteron receptor

cyt: cytoplasmic; staining; med: medium, str: strong

CHAPTER 4

DISCUSSION

4.1 *mdr1* Gene Expression in Tumor Tissues of Breast Cancer Patients

In this study, *mdr1* gene was shown to be involved in clinical resistance in locally advanced breast cancer patients. Its expression during chemotherapy resulted in clinical resistance. *Mdr1* gene expression started in 80% (4/5) of the clinically resistant patients during chemotherapy where as none of the 20 patients that responded to neoadjuvant chemotherapy had *mdr1* gene expression induced during chemotherapy. The only exception was a patient in the clinically responsive group who had persistent *mdr1* gene expression during chemotherapy. The fifth patient with clinical resistance showed *mdr1* gene expression existing before chemotherapy and persisting after chemotherapy as well. Among the drugs used in this group of patients, anthracyclines such as doxorubicin and epirubicin most probably were responsible for the induction of the gene expression.

In the previous studies, various molecular methods were used for the detection of *mdr1* gene expression such as Northern blotting (Goldstein LJ *et al.*, 1989; Keith and Brown, 1990; Wallner J *et al.*, 1991, Merkel DE *et al.*, 1989), in-situ hybridization (Kacinski BM *et al.*, 1989) and RT-PCR (Chevillard S *et al.*, 1997; Charpin C *et al.*, 1994; Wang CS *et al.*, 1997; Faneyte IF *et al.*, 2001; Lacave R *et al.*, 1998; Dexter DW *et al.*, 1998; Filipits M *et al.*, 1996; Arnal M *et al.*, 2000). Besides, the patients selected for the studies formed heterogenous groups including healthy volunteers, those with benign breast diseases, and both early and locally advanced stage breast cancer patients. In addition, gene expression was studied usually from a single sample obtained from the patient either at the time of

diagnosis or before chemotherapy for metastatic disease. In most of the studies tissue samples from primary breast cancer patients were used. The results could only give information about the prevelance of *mdr1* gene expression in untreated patients. In the previous studies, the reported values of *mdr1* gene expression ranged between 15-100%. The results of these studies are shown in Table 4.1. Among the previously utilized methods, RT-PCR was found to be the most sensitive one, since, even a few cancer cells with *mdr1* expression could be detected by this method. Thus, the detection rates of *mdr1* gene expression increased to 60-100% when RT-PCR was used.

Table 4.1. Results of the studies evaluating *mdr1* gene expression

Author	Tumor Type	*Expression Ratio	% Expression
Goldstein et al. (1989)	PBC	9/57	15
Keith et al. (1990)	PBC	25/49	51
Kacinski et al. (1989)	PBC	9/16	56
Wallner et al. (1991)	PBC	27/61	44
Charpin et al. (1994)	PBC	11/14	81
Wang et al. (1997)	PBC	44/52	84
Faneyte et al. (2001)	PBC	52/52	100
Chevillard et al. (1997)	PBC	18/64	28
Lacave et al. (1998)	PBC	61/74	82
Dexter et al. (1998)	PBC	74/74	100
Filipits et al. (1996)	PBC	80/134	60
Chevillard et al. (1997)	LABC	2/17	12
Arnal et al. (2000)	LABC		
Before CT		38/40	95
After CT		40/40	100
This study (2004)	LABC		
Before CT		3/25	12
After CT		6/25	24

PBC: primary breast cancer, samples taken before chemotherapy

LABC: locally advanced breast cancer; CT: chemotherapy

*Expression ratio: number of patients expressing *mdr1* gene / total number of patients

In the previous studies where Northern blotting method was used to detect the gene expression, the patients were selected among primary breast cancer patients with no previous drug treatment. Among these studies, except one (15%) (Goldstein LJ *et al.*, 1989), *mdr1* gene expression was found to be present in about half of the patients (range 46-56%). However, in the studies utilizing RT-PCR for the detection of the gene, *mdr1* gene expression was over 60% in primary breast cancer patients. Even, Dexter et al. (1998) depicted *mdr1* gene expression in all of 74 patients. Similarly, Faneyte et al. (2001) detected *mdr1* gene expression in all of their patients, but the expression intensity was low. These moderate to high rates of *mdr1* gene expression are thought to be in accordance with the clinical observations of intrinsic resistance in breast cancer.

In the current study, a more homogenous group of patients were selected among the locally advanced breast cancer patients (stage IIIB) and, especially, the initiation of *mdr1* gene expression during chemotherapy was evaluated for its possible role in clinical response. Each patient had tissue samples obtained both before and after chemotherapy enabling us to directly evaluate the role of *mdr1* gene expression in developing resistance. In addition, the most sensitive method, namely RT-PCR, was used for the detection of *mdr1* gene expression. This type of study design helped us to depict the possible role of *mdr1* gene in clinical resistance.

Previous studies, including only the patients with locally advanced breast cancer as in this study, reported equivocal results (Chevillard S *et al.*, 1997; Arnal M *et al.*, 2000; Burger H *et al.*, 2003). Chevillard et al. (1997) found *mdr1* gene expression as low as 12% in their untreated stage III A and B patients. In contrast to this, Arnal *et al.* (2000) detected the expression of *mdr1* gene before chemotherapy in 95% (38/40) of the patients with locally advanced breast cancer including inflammatory cancer cases. After giving a chemotherapy protocol including doxorubicin, *mdr1* gene expression was found to be present in all of the patients. Thus, no difference with regard to *mdr1* gene expression was recorded between clinically responsive and unresponsive patients. This result was due to the high expression rate of *mdr1* gene before chemotherapy. In contrast, the patients in this study had lower *mdr1* expression ratio (12%) before chemotherapy and the increase in the expression ratio (24%) found after chemotherapy was significant ($p<0.001$).

Similar to the results of the current study, Burger *et al.* (2003) reported a correlation between *mdr1* gene expression and clinical response to chemotherapeutic drugs. *Mdr1* gene was expressed in 68% of the clinically resistant patients whereas the expression rate was only

17% in clinically responsive patients and this difference between the groups reached statistical significance. In the current study, *mdr1* gene expression was detected only in 12% of the patients before chemotherapy. The rate of expression increased to 24% after chemotherapy. The expression rate of *mdr1* gene was 100% in clinically resistant patients and in 80% of these patients *mdr1* gene expression was induced during chemotherapy. On the other hand, only 5% of the clinically responsive patients had *mdr1* gene expression. This difference in expression between the two groups reached statistical significance ($p<0.001$).

As *mdr1* gene expression is shown to confer clinical resistance in locally advanced breast cancer patients, application of chemotherapy protocols excluding MDR phenotype related drugs, such as anthracyclines, taxanes, or vinca alkaloids to *mdr1* expressing patients could be a logical solution to overcome this type of drug resistance.

4.2 *mrp1* Gene Expression in Tumor Tissue of Breast Cancer Patients

Studies on *mrp1* gene expression in breast cancer patients are rare compared to those on *mdr1* gene expression probably due to the more recent discovery of *mrp* gene family. Due to the sensitivity of RT-PCR method in recent years, almost all of the previous studies utilized this method to evaluate *mrp1* gene expression. *mrp1* expression changed between 70-100% in the previous studies. The results of the previous studies are given in Table 4.2. The *mrp1* gene expression rate of 68% detected before chemotherapy in this study is compatible with the previous studies. *Mrp1* expression increased to 84% after chemotherapy in this study.

Burger et al. (2003) included patients with advanced breast cancer evaluating the role of *mrp1* gene in clinical resistance. Although *mrp1* gene expression was reported to have an inverse relation with the clinical response of the patients to chemotherapy, this difference was not statistically significant. Also in this study, *mrp1* gene expression was not shown to correlate with the clinical response of the patients. Expression rate of *mrp1* gene was even slightly higher in clinically responsive patients compared to clinically unresponsive patients (85% vs 80%). Although the percentage of stained cells with MRP1 antibodies frequently increased after chemotherapy in patients with clinical resistance, a statistical difference was not detected due to the high incidence of *mrp1* gene expression in clinically responding patients as well. Thus, the expression of *mrp1* gene could be a result of changes occurring during malignant transformation in breast cells rather than a response to cytotoxic drugs. On the other hand, it may well be a sign of intrinsic chemoresistance of breast cancer. The

expressions of various oncogenes and suppressor genes change during transformation to malignancy. Some of these genes may well have a role in the regulation of *mrp1* gene and increased expression may result from the interaction between these genes.

Table 4.2. Results of the studies evaluating *mrp1* gene expression

Author	Tumor Type	*Expression Ratio	% Expression
Filipits et al. (1996)	PBC	134/134	100
Ito et al. (1998)	PBC	19/27	70
Lacave et al. (1998)	PBC	74/74	100
Dexter et al. (1998)	PBC	74/74	100
This study (2004)	LABC		
Before CT		17/25	68
After CT		21/25	84

PBC: primary breast cancer, samples taken before chemotherapy

LABC: locally advanced breast cancer; CT: chemotherapy

*Expression ratio: number of patients expressing *mdr1* gene/total number of patients

4.3 Immunohistochemistry Staining for P-glycoprotein

In order to support the expression of the genes involved in drug resistance mechanisms, presence of the protein products of these genes in the breast cancer tissues should be shown (Beck WT *et al.*, 1996). The protein product of *mdr1* gene, or Pgp, was frequently evaluated in the previous studies. IHC method was usually used to show Pgp. This method was chosen for its widespread availability, lower cost, ease of application, and possibility for retrospective analysis of paraffin embedded tissues. On the other hand, lesser sensitivity and specificity, and observer dependence may be stated as the disadvantages of this method.

In the previous studies, various monoclonal antibodies were used to detect Pgp. C219, C494, MRK16, MRK17, UIC2, and JSB1 are among the frequently used antibodies. As newer and more specific antibodies were developed, the detection sensitivities of Pgp increased in

parallel. In a previous study comparing the effectivity of monoclonal antibodies specific for Pgp, JSB1 was found to be superior to other antibodies (Linn SC *et al.*, 1997). For this reason, JSB1 was chosen for the detection of Pgp in this study.

As it is seen from Table 4.3, previous studies have different results regarding Pgp presence in breast cancer tissues. Pgp positivity was reported to change between 0-100% similar to the studies on *mdr1* gene expression. As in case of all of the studies on MDR mechanisms, the variation in the patient groups and the monoclonal antibodies used could be the reason for this discrepancy.

When the studies including locally advanced breast cancer patients were seperately evaluated, the positivity rate did not show any difference compared to primary breast cancer patients, changing between 0-100%. However, a detailed analysis of these studies revealed Pgp positivity to be between 0-85% in untreated patients.

Among these studies, three had evaluated Pgp positivity both before and after chemotherapy. Vargas-Roig et al. (1999) detected Pgp positivity in 59% (22/37) of the breast cancer patients before chemotherapy. After chemotherapy, 43% of the patients had persistent Pgp positivity where as 16% of the patients became Pgp negative. In addition, none of the patients showed Pgp positivity induced during chemotherapy. Similar to this case, two of the Pgp positive patients in this study (P 3 and P 16) became Pgp negative after chemotherapy although they were slightly positive before chemotherapy. This could be due to false positivity. These two patients had clinical response to chemotherapy.

Linn et al. (1997) evaluated 40 locally advanced breast cancer patients in their study. However, among these only 17 patients had both pre- and post-chemotherapy tissue samples. Pgp positivity rate was 65% before chemotherapy and this rate decreased to 59% after chemotherapy in contrast to the expectation of Pgp induction. In addition, Pgp results were not correlated with clinical response to chemotherapy. Thus, a firm conclusion regarding the role of Pgp in clinical response can not be reached yet. However, some examples in this study show agreement with Pgp status to change from positive to negative during chemotherapy.

In contrast to the previously mentioned two studies, Rudas et al. (2003) reported a significant increase in Pgp positivity during chemotherapy. The rate of Pgp positivity increased from

Table 4.3. Results of the studies evaluating P-glycoprotein in breast cancer patients

Author	Tumor Type	*Pgp Positivity Ratio	% Positivity	Monoclonal Antibody
Sugawara et al. (1988)	PBC	9/57	15	MRK 16
Merkel et al. (1989)	PBC	0/125	0	C219
Bodey et al. (1997)	PBC	14/15	93	JSB1, C219
Sanfilippo et al. (1991)	PBC	10/34	29	C219
Charpin et al. (1994)	PBC	113/213	53	JSB1
Faneyte et al. (2001)	PBC	44/62	71	JSB1
Wang et al. (1997)	PBC	44/52	84	JSB1, C494
Dexter et al. (1998)	PBC	2/31	6	JSB1
Filipits et al. (1996)	PBC	36/63	57	C219
Linn et al. (1997)	PBC	2/20	10	JSB1, C219
Dixon et al. (1992)	LABC	0/26	0	C219
Verelle et al. (1991)	LABC	17/20	85	C494
Botti et al. (1993)	LABC	19/25	76	C219
Ro et al. (1990)	LABC	20/40	50	C219
Vargas-Roig et al. (1999)	LABC			JSB1, C494
Before CT		22/37	59	
After CT		16/37	43	
Linn et al. (1997)	LABC			JSB1, C219
Before CT		11/17	65	
After CT		10/17	59	
Rudas et al. (2003)	LABC			JSB1
Before CT		37/68	55	
After CT		68/68	100	
This study (2004)	LABC			JSB-1
Before CT		4/25	16	
After CT		9/25	36	

PBC: primary breast cancer; LABC: locally advanced breast cancer; CT: chemotherapy

*Pgp positivity ratio: number of positively stained patients / total number of patients

55% to 100% after chemotherapy. Although a significant increase in Pgp positivity was detected indicating an induction of *mdr1* gene, no correlation between the clinical response of the patients and Pgp positivity was found. This finding was in contrast to the results of the current study. Pgp positivity detected during chemotherapy significantly affected clinical drug response to chemotherapy. All of the clinically unresponsive patients had Pgp positivity in their tissue samples where as only 10% of the clinically responding patients had Pgp positivity.

Generally, IHC results for Pgp were in parallel with RT-PCR results for *mdr1* gene in this study. After chemotherapy, seven patients had Pgp positivity detected with IHC and two of these patients did not have *mdr1* gene expression. These two patients were in the clinically responsive group and this difference between RT-PCR and IHC methods supports the higher sensitivity and specificity of RT-PCR. Since IHC is a cheaper and a widely available method, it could be prefferred to RT-PCR in clinical practice although it is less accurate.

4.4 Immunohistochemistry Staining for MRP1

In the previous studies, the presence of MRP1 was evaluated in conjunction with *mrp1* gene expression or alone. Various monoclonal antibodies were utilized such as MRPr1, MRPm6, QCRL-1, and QCRL-3 depending on the institution. In this study, QCRL-1 antibodies were used.

The detection rate of MRP1 in breast cancer tissues varied between 18-100% depending on the monoclonal antibodies used and patient groups studied. Even in primary breast cancer patients, MRP1 was found to be present in the tissues indicating this as an intrinsic property of breast cancer. The results of the previous studies evaluating MRP1 in breast cancer are depicted in Table 4.4.

In the studies with locally advanced breast cancer patients, conflicting results were reported. Linn et al. (1997) found MRP1 positivity in 59% of the patients before chemotherapy. This rate did not show any significant difference after chemotherapy, 53% MRP1 positivity.

On the other hand, Rudas et al. (2003) reported MRP1 positivity in 62% of the locally advanced breast cancer patients before chemotherapy. The MRP1 positivity was 88% after chemotherapy and the difference between before and after chemotherapy was significant.

Similar to this, MRP1 staining was abundantly present in 68% patients before chemotherapy in the current study. MRP1 positivity increased to 84% after chemotherapy. Although MRP1 positivity increased during chemotherapy indicating increased expression of *mrp1* gene, the difference was not statistically significant. The reason for this could be widespread presence of MRP1 among breast cancer patients.

When the two methods utilized in this study were compared, IHC method was found as accurate as RT-PCR in detection of *mrp1* overexpression. IHC could be the method of choice to study the drug resistance resulting from *mrp1* expression due to its availability and cost-effectiveness.

Table 4.4. Results of the studies evaluating MRP1 in breast cancer patients

Author	Tumor Type	*MRP1 Positivity Rate	% Positivity	Monoclonal Antibody
Filipits et al. (1996)	PBC	63/63	100	QCRL-1/3
Nooter et al. (1995)	PBC	2/11	18	MRPr1
Nooter et al. (1997)	PBC	87/259	34	MRPr1
Linn et al. (1997)	PBC	16/20	80	MRPr1
Linn et al. (1997)	LABC			MRPr1
Before CT		10/17	59	
After CT		9/17	53	
Rudas et al. (2003)	LABC			MRPr1
Before CT		42/68	62	
After CT		60/68	88	
This study (2004)	LABC			QCRL-1
Before CT		17/25	68	
After CT		21/25	84	

PBC: primary breast cancer; LABC: locally advanced breast cancer; CT: chemotherapy
*MRP1 positivity ratio: number of positively stained patients / total number of patients

4.5 Clinical Response to Chemotherapy

Resistance to chemotherapy is an important issue for all types of cancers. Drug resistance can either be an intrinsic or an acquired form of resistance. In intrinsically resistant tumors, MDR related genes are constitutively expressed and these are the malignancies of tissues where these genes have a role in secretion or protection. On the other hand, acquired resistance can either be due to selection of drug resistant clones during chemotherapy or a real induction of MDR related genes in cancer cells. In cancer types with intrinsic resistance, chemotherapy is usually not considered as a treatment option and other modalities such as surgery or radiotherapy are utilized, if possible. However, in cancers showing induced type of resistance to chemotherapy, initially the tumor is chemosensitive. Later in the course of the disease, resistance may develop disabling us to properly treat the recurrent or the metastatic disease with chemotherapy.

Among various cancers, breast cancer is thought to be moderately responsive to chemotherapy. However, to obtain a sustained cure with the combined use of surgery, chemotherapy, radiotherapy, and hormonotherapy is not always possible since local or distant disease recurrences are observed many years after the treatment of the primary tumor. Then, chemoresistance becomes a real challenge to overcome especially in the unresponsive group of patients. Knowing the probability of developing drug resistance may help us to choose the type of drugs to be used in advance. In addition, drugs beneficial in the reversal of MDR mechanisms could be added to the standard chemotherapy.

The relationship between clinical response to chemotherapy and MDR in breast cancer patients has been studied previously. However, conflicting results have been reported. There are a few reasons for this discrepancy. First, the patients with recurrent or metastatic disease were included as clinically resistant patients and their tissue samples were analyzed retrospectively in search for the expression of MDR related genes. This patient selection method could only help to depict the intrinsically resistant patients with the higher probability of treatment failure. Thus, Burger et al. (2003) included patients with advanced disease in their study. In this group of patients, both *mdr1* and *mrp1* gene expressions prior to chemotherapy decreased clinical response. However, this difference between clinical responders and non-responders reached significant levels only for *mdr1* gene.

Second, MDR related genes were studied in locally advanced breast cancer patients only prior to chemotherapy. The gene expression results were compared in clinically sensitive and

resistant patient groups. Again, this type of study design can only show the prevalence of MDR related genes and points to the intrinsic properties of the patients. Ro et al. (1990) reported Pgp positivity as 29% in objective response to chemotherapy group and this rate increased to 100% in progressive disease group. Similarly, Verelle et al. (1991) found a decrease in clinical response in patients with strong Pgp positivity. Besides, Botti et al. (1993) observed that all patients with recurrent disease had Pgp positivity where as 43% of the patients without any evidence of recurrent disease stained negative for Pgp. In general, these studies indicate that Pgp positivity may have a role in clinical resistance to chemotherapy. However, chemoresistance is not detected in all of the patients expressing MDR related genes rejecting a definite role. There is evidence in the previous and the current study showing that in some patients, gene expression may decrease or even stop during chemotherapy. As gene expression was studied only prior to chemotherapy, it is impossible to exactly know the changes in gene expression status during chemotherapy. The patients with MDR related gene expression before chemotherapy and responded to chemotherapy may well have no gene expression after chemotherapy.

For the above mentioned reasons, instead of detecting the drug resistant patients prior to chemotherapy, we need to find the patients with induced gene expression. In order to detect the gene induction, at least two tissue samples must be used, one obtained before and the other after chemotherapy. Also, the drugs known to be transported by MDR related pumps should be used for chemotherapy. By this way, it may be possible to prove the role of MDR related genes in clinical resistance in breast cancer. Although Rudas et al. (2003) reported an induction of both *mdr1* and *mrp1* genes during chemotherapy, a correlation between gene expression and clinical resistance could not be shown. In contrast to this, Arnal et al. (2000) depicted the induction of *mdr1* gene expression to be related to clinical resistance to chemotherapy. Similar to this study, a correlation was found between *mdr1* gene induction during chemotherapy and clinical resistance of the patients in the current study. However, *mrp1* gene induction was not shown to be related to clinical resistance.

4.6 Relationship Between Oncogenes/Tumor Suppressor Genes and Multidrug Resistance

Different cancer tissues may exhibit various levels of multidrug resistance gene expression. High levels of expression could be detected before chemotherapy even the original tissue is known to be non-resistant to drugs. Hence, constitutive *mdr1* or *mrp1* gene expression may

be regulated by other genes involved in malignant transformation. Altered expression of proteins controlling cell growth and death can adversely affect drug therapy by either changing individual cell's ability to respond to death signals or influencing transcription of drug resistance genes.

The first evidence that another gene could affect the expression of drug resistance genes came from an observation showing that wild-type p53, a tumor suppressor gene highly mutated in various cancers, repressed transcription of *mdr1* gene (Zastawny RL *et al.*, 1993). This interaction between the two genes is reported to be by direct binding of p53 to *mdr1* gene promoter (Johnson RA *et al.*, 2001). However, in cancer cells, mutant forms of p53 with loss of known functions are detected in higher amounts. Thus, *mdr1* gene might be activated in cancer due to a decrease in the repressor effects of p53. Supporting these findings, an inverse relation between *mdr1* gene and mutated p53 positivity was also reported in a clinical study (Ferrero JM *et al.*, 2000).

Similarly, wild-type p53 has been shown to repress the transcription of *mrp1* gene (Bahr O *et al.*, 2001). It is reported to be due to a direct interaction between p53 and *mrp1* gene. It is possible that mutant form of p53 will have less effect on *mrp1* gene transcription leading to a higher rate of expression. As a result, this may explain the high rate of *mrp1* expression in breast cancer tissues both in the previous studies and the current study. Malignant transformation itself could be the underlying reason for *mrp1* gene expression rather than an induction due to chemotherapeutic drugs. In contrast to these previously reported findings, no correlation was detected between both *mdr1* and *mrp1* gene expressions and p53 positivity in the current study. Similar to this, Chevillard et al. (1997) reported no correlation between *mdr1* gene and mutant p53 positivity.

Besides, no relation was shown between both *mdr1* and *mrp1* gene expressions and c-erb-b2, an epidermal growth factor that is constitutively activated after malignant transformation in various cancers. Besides, the studied two genes had no relation to both estrogen and progesteron receptors in this study. Wallner et al. (1991) reported similar findings and found no correlation between *mdr1* gene expression and steroid hormone receptors and c-erb-b2 positivity.

In addition, *mdr1* gene is known to be a target of ras/raf signalling pathway. Previous studies indicated that signalling by ras converges on the GC-rich binding site for transcription factors Sp1 and Sp3, located within *mdr1* promoter. A similar GC element is also present in

the promoter regions of several MRP genes. The regulation of *mdr1* gene expression by ras/raf system probably involves a complex interaction of different transcription factors within a very discrete promoter region. Recently, activation of phospholipase C has been reported to activate *mdr1* gene transcription. Constitutive coexpression of raf enhances transcription, suggesting that phospholipase C delivers ras/raf signal to the *mdr1* promoter through a distinct binding site.

Besides tumor suppressor genes, oncogenes may as well be involved in the transcriptional regulation of multidrug resistance genes. Recently, a transcription factor complex, namely AP-1, was shown to affect *mdr1* gene transcription. AP-1 is, in fact, the general term for transcription factor complexes composed of members of Fos and Jun oncogene families. Previously, elevated levels of c-fos have been demonstrated in a number of drug-resistant cell lines. Besides, c-jun was found to be associated with an increase in *mdr1* gene expression at the mRNA level.

4.7 Future Perspectives

There is an immense amount of investments and investigations going on in various fields of research for the treatment of cancer. Generally, a consensus has been developing indicating that the treatment should be individualized in the coming years. In order to treat the patient, biological characteristics of the tumor must be clearly known. Recent developments in microarray technology will probably help to plan the treatments for the individual patients.

Cancer prevention strategies, including the replacement of mutated genes by wild-type genes or decreasing the effects of mutated genes on the organism, will probably have a priority in research field since with these strategies healthy individuals will be spared from the deleterious consequences of cancer. In addition to the prevention strategies, methods of increasing the effect of known treatment modalities are a part of the ongoing research. In this respect, new strategies to overcome drug resistance will be beneficial.

Targeted drug therapy may be one of the methods to be used to increase the efficacy of chemotherapeutic drugs. This strategy will increase the drug concentration at the tumor site and prevent the unwanted systemic side effects of the drugs. Thus, smaller doses would be administered with higher response rates. In addition, drugs developed for the reversal of MDR can be targeted to tumor cells. In the previous studies, many pharmacological agents

have been tried in order to reverse the multidrug resistance, but a definite benefit has not been reported in experimental and clinical studies. Still, the research is going on for new reversal agents and their clinical applications.

Another strategy to increase the drug efficacy may be to manipulate MDR genes, if their role is thought to be the underlying cause detected in the individual patient. Gene manipulation can be done at the transcription or the translation level. So it is important to evaluate the regulatory factors for the transcription of these genes for further manipulations. On the other hand, control over the translation level can be achieved by blocking mRNAs with anti-sense codons. Thus, the production of active proteins could be prevented with this method.

Although the research for developing newer strategies in the treatment of cancer is highly expensive and time consuming, a value cannot be given for the benefits obtained from this research.

4.8 Conclusion

In this study, *mdr1* gene expression was most probably induced by anthracyclines such as doxorubicin and epirubicin. Thus, administration of chemotherapy regimens excluding MDR phenotype related drugs, such as anthracyclines, taxanes, or vinca alkaloids to *mdr1* expressing patients would be a logical solution to overcome the drug resistance.

In addition, new treatment strategies could be developed against multidrug resistance. New drugs could be used for the reversal of these drug efflux pumps. Also, chemotherapy drugs could be targeted to MDR genes expressing tumor cells increasing the response obtained from these drugs. In addition, gene therapy could be used to block MDR genes at the transcriptional and translational levels.

When two methods used in this study, RT-PCR and IHC, are compared, the results were almost similar. The widespread availability, lower cost, ease of application, and the possibility of retrospective analysis favor the use of IHC in the studies regarding MDR.

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APPENDIX A

COMPOSITIONS OF BUFFERS AND SOLUTIONS

Guanidium thiocyanate (GTC) solution

- 4 M Guanidium thiocyanate
- 25 mM Sodium citrate
- 0.5% N-lauroylsarcosine
- 0.1 M 2-mercaptoethanol

Digestion Buffer

- 10 mM Tris-HCl
- 100 mM NaCl
- 0.5% SDS
- 25 mM EDTA

Loading Solution

- 0.25% Bromophenol blue
- 0.25% Xylene cyanol
- 50% Glycerol
- 1 mM EDTA

1 X TE (pH 8.0)

- 10 mM Tris-HCl
- 1 mM EDTA

10 X MOPS (pH 7.0)

- 0.2 M MOPS
- 50 mM Sodium acetate
- 10 mM EDTA

50 X TAE (pH 7.6)

- 2 M Tris base
- 1 M Acetic acid
- 50 mM EDTA

10 X TBE (pH 8.3)

- 0.89 M Tris base
- 0.89 M Boric acid
- 20 mM EDTA

PCR Reaction Buffer

- 670 mM Tris-HCl (pH 8.8)
- 100 mM 2-mercaptoethanol

PBS

One PBS tablet per 100 ml H_2O

APPENDİX B

PERCENTAGE OF CELLS STAINED WITH MRP1

Table B.1. The total number of stained cells per total number of counted cells for MRP1 both before and after chemotherapy

Patient	Before Chemotherapy	After Chemotherapy
P 1	--	--
P 2	187/312 = 60 %	231/330 = 70 %
P 3	240/342 = 70 %	304/320 = 95 %
P 4	91/314 = 30 %	257/286 = 90%
P 5	89/296 = 30 %	242/242 = 100 %
P 6	30/298 = 10 %	64/318 = 20 %
P 7	31/312 = 10 %	262/328 = 80 %
P 8	228/326 = 70 %	214/214 = 100 %
P 9	173/288 = 60 %	188/188 = 100 %
P 10	--	--
P 11	83/276 = 30 %	228/228 = 100 %
P 12	136/272 = 50 %	112/280 = 40 %
P 13	--	123/308 = 40 %
P 14	--	80/266 = 30 %
P 15	--	--
P 16	28/284 = 10 %	293/326 = 90 %
P 17	186/266 = 70 %	212/212 = 100 %
P 18	254/254 = 100 %	220/220 = 100 %
P 19	244/244 = 100 %	232/232 = 100 %
P 20	198/198 = 100 %	206/206 = 100 %
P 21	202/202 = 100 %	226/226 = 100 %
P 22	--	--
P 23	29/294 = 10 %	277/308 = 90 %
P 24	--	202/252 = 80 %
P 25	--	26/260 = 10 %

VITA

Mustafa Can Atalay was born in Mersin on February 22, 1966. He received his M.D. degree in Faculty of Medicine from Hacettepe University in July 1990. He completed his residency at Ministry of Health Ankara Hospital and became a general surgeon in December 1994. Since then he has been working at Ministry of Health Ankara Oncology Hospital. His main area of interest is cancer research.