GLUTATHIONE S-TRANSFERASE PI EXPRESSION IN INVASIVE DUCTAL BREAST CARCINOMA

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SUMMMARY

This study involved invasive ductal breast cancers from 137 female patients with no distant metastasis on diagnosis and no neo-adjuvant chemotherapy prior to surgery. Focus was placed on the expression of glutathione S-transferase pi (GST-pi), a Phase II detoxification enzyme that has recently been implicated in protection against apoptosis. GST-pi expression was evaluated in conjunction with the expression of biological markers, namely Bcl-2, metallothionein (MT), p-glycoprotein (Pgp) and Y-box binding protein-1 (YB-1), as well as apoptosis detected by the TUNEL method. It was further correlated with measurements of total GST activity and levels of oxidative stress by quantification of TBARS. Clinical significance of the expression of the biological markers was examined using known clinico-pathological parameters and early recurrence on follow-up.

GST-pi expression was detected in 58%, Bcl-2 expression in 37%, MT expression in 88%, and Pgp expression in 43% of the breast cancers. YB-1 expression was detected in 95% and 100% of tumours, using 2 different antibodies, Frgy-1 and Ckyb-1, respectively. In most GST-pi positive/Bcl-2 positive tumours, there was a distinct accumulation of GST-pi within the nucleus of cancer cells when examined by double immunofluorescence labeling under confocal microscopy. GST-pi expression was associated with Pgp expression ($p=0.033$) and higher levels of YB-1 immunoreactivity ($p=0.048$). A direct interaction between YB-1 and Pgp was demonstrated using the computer-based Resonant Recognition Model.

Univariate analysis revealed that GST-pi positive, Bcl-2 positive, and lower histological grade tumours had decreased levels of apoptosis ($p=0.024$, $p=0.011$, and

p=0.029, respectively). However, multivariate analysis showed that histological grade and Bcl-2, but not GST-pi immunoreactivity, were correlated with apoptotic status. Apoptosis in GST-pi negative tumours was not correlated with GST activity but GST-pi positive tumours within the same range of oxidative stress showed a reduction in apoptosis with increased GST activity. This correlation was absent in GST-pi positive tumours experiencing higher oxidative stress. It appeared that GST-pi expression may influence the level of GST activity and delay apoptosis in breast cancer, although its expression in tumours with higher levels of oxidative stress may not be sufficient in abrogating the deleterious effects. Whilst GST-pi immunoreactivity was not significantly correlated with any of the traditional histologic factors known to influence prognosis, multivariate analysis showed that GST-pi expression, higher MT expression and Bcl-2 negative tumours have significantly increased recurrence risk.

Considering the group of patients who received adjuvant chemotherapy, disease-free survival in patients with GST-pi–positive tumours was worse than that in patients with GST-pi–negative tumours (p=0.04). It was also worse in patients with higher MT expression compared to those with lower MT expression (p=0.048). Interestingly, we found that patients who were on a chemotherapy regime which contained an anthracycline (a PGP substrate) and subsequently developed recurrence, had a higher YB-1 score compared to patients on the Cyclophosphamide/Methotrexate/5 Fluorouracil regime (p=0.024).

In conclusion, GST-pi expression is associated with more aggressive tumours and this effect may be partly explained by protection against oxidative stress and apoptosis. Further, MT and YB-1 show promise as biological markers of chemotherapy resistance.

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LIST of ABBREVIATIONS

AJCC	American Joint Committee on Cancer
Apaf-1	apoptotic protease activating factor 1
APES	3-aminopropyl-tri-ethoxysilane
ARE	anti-oxidant responsive element
ATM	ataxia telangiectasia mutated
Bax	Bcl-2-associated X protein
Bid	BH3 (Bcl-2 homology region-3) interacting domain death agonist
BRCA1	breast cancer antigen 1
BRCA2	breast cancer antigen 2
BSA	bovine serum albumin
CDNB	1,2-chloro-2,4-dinitrobenzene
CEA	carcino-embryonic antigen
Ckyb-1	anti-chicken YB-1 antibody
CMF	cyclophosphamide/methotrexate/5-fluorouracil chemotherapy
CRE	cAMP responsive element
Cy3	cyanine-3
DcR3	decoy receptor 3
DDT	dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
E2F1	E2 promoter binding factor 1
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
FasL	Fas ligand
FITC	fluorescein isothiocyanate
Frgy-1	anti-frog YB-1 antibody
GADD45	growth arrest and DNA-damage-inducible 45
GST	glutathione S-transferase
IAP	inhibitor of apoptosis proteins
Ile	isoleucine
JNK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated kinase kinase
MAPKKK	mitogen activated kinase kinase kinase
MDR1	multidrug resistance protein 1
MLH1	MutL protein homolog 1
MOAT	multispecific organic anion transporter
MRP1	multi-drug resistance associated protein 1
MSH2	MutS homolog 2
MT	metallothionein
NCBI	National Centre for Biotechnology Information
NK cells	natural killer cells

PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDK1	3-phosphoinositide dependent protein kinase-1
Pgp	p-glycoprotein
PI3K	phosphotidylinositol 3 kinase
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma
ROS	reactive oxygen species
RRM	Resonant Recognition Model
SDS	sodium dodecyl sulfate
SMAC	second mitochondria-derived activator of caspase
TBARS	thiobarbituric acid reactive substances
TGF	transforming growth factor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRADD	TNF-associated death domain
TRAIL	TNF-related apoptosis inducing ligand
TRE	12-O-tetradecanoylphorbol-13-acetate (TPA) response element
TUNEL	deoxynucleotidyl transferase-mediated, dUTP-biotin nick end-labeling
Val	valine
YB-1	Y-box binding protein-1

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Introduction

Breast cancer commonly refers to the uncontrolled and malignant proliferation of epithelial cells from ducts or lobules of the breast. It typically presents in a woman with a painless breast lump of variable duration. In the pre-menstrual woman, the lump will persist through the menstrual cycle. Symptoms of pain, nipple retraction, nipple discharge, skin retraction, axillary mass (due to axillary lymph node enlargement from metastasis) may be present. With breast cancer screening, many breast cancers are discovered in the form of abnormal mammography in an asymptomatic woman.

1.1 History of breast cancer

We have come a long way since the earliest records of its diagnosis in the ancient Egyptian era of about 2500 BC. Over time, ideas about its pathogenesis and treatment have evolved.

The first known description of breast cancer was recorded in the Edwin Smith papyrus where a male patient had features characteristic of breast cancer (Breasted, 1930). Surgeons then recognized that little could be done for these tumours. Hippocrates (460-370 BC) felt that breast cancer was due to the cessation of menstrual flow, leading to the subsequent imbalance and engorgement of the breast. He was of the opinion that "treatment causes speedy death, but to omit treatment is to prolong life". Later, Caudius Galen (129-c.200 AD), a Greek physician and philosopher, suggested that melancholia was the chief factor in the development of breast cancer. He felt that excision could not correct the physical imbalance, and it was to be treated with special diets and concoctions.

In spite of these medical theories, breast surgery was still being performed and progress was made. As early as the first century AD, Aulus Cornelius Celsus suggested that early cases of breast cancer would respond to intervention. (De Moulin, 1983).

In the 1600s, surgeons began to attempt the removal of axillary lymph nodes with the understanding that breast cancer could spread to the lymph nodes and subsequently to other organs. Henry Francois LeDran (1685-1770) noted that poor prognosis was associated with metastatic lymph node deposits. Throughout the 1800s, breast cancer surgery with axillary clearance slowly evolved, helped by developments in anaesthesia and anti-sepsis. In 1894, William Halsted (1852 - 1922) of Johns Hopkins Hospital reported a substantial survival improvement with a radical mastectomy that involved the removal of the pectoralis major (Halsted, 1894). Subsequently, David Patey (1899 - 1977) devised a modified technique that preserved the pectoralis major; the pectoralis minor was instead resected to facilitate lymph node dissection (Patey, 1948). This method, still widely practiced currently, reduced the mobidity experienced by women after radical mastectomy, with no compromise in survival (Maddox *et al.*, 1983).

At about the same time in 1895, X-rays were discovered by Wilhelm Conrad Rontgen (1845-1923) and Hermann Gocht (1860-1983) first reported its successful use in 2 cases of locally advanced breast cancer in 1897.

Later, it became evident that surgery could be coupled with other adjuvant treatments for greater benefit. The concept that breast cancer is a systemic disease and cure depends on systemic anti-cancer treatment in addition to local treatment became more widely accepted.

Hormonal treatment became popular after surgical and radiation castration improved the clinical course of breast cancer patients in the late 1800s to early 1900s (Beatson, 1896). Eventually, tamoxifen, an anti-estrogenic drug and the mainstay of hormonal treatment for breast cancer today, emerged in the 1970s (Ward, 1973). Experiments on cytotoxic chemotherapy for breast cancer only began in the 1960s, initially with single agents, subsequently with various drug combinations.

Today, surgery, radiotherapy, cytotoxic chemotherapy and hormonal drugs are well established treatment modalities for breast cancer. Yet, the story of breast cancer is still not complete. Since the turn of the century, more than 7000 papers on breast cancer are added to the Medline, reflecting the intense activity of scientists all over the world, trying to understand breast cancer and fine-tune its treatment.

1.2 Epidemiology

Breast cancer is the most common cancer and the second leading cause of cancer deaths in women around the world. It is predominantly a female disease, occurring more frequently in women than in men in the ratio of 130:1. The older a woman is, the greater her chances of developing breast cancer - approximately three quarters of breast cancer cases occur in women over 50 years of age (Feuer *et al.*, 1999).

One in 8 American women will have breast cancer in her lifetime. The World Health Organisation estimated that 55,900 new cases of breast cancer were diagnosed in Southeast Asia alone in the year 2000 and was responsible for 25,000 deaths in the same

region during that time (Ferlay *et al.*, 2001). Breast cancer constitutes 23% of all cancers diagnosed in women in Singapore and its incidence is rising. In the years 1993 to 1996, the incidence rate of breast cancer in Singapore increased by an average of 3.68% per year (Chia *et al.*, 2000).

Breast cancer incidence varies across geographic regions and ethnic groups. Populations of the same ethnic origin living in different countries have different breast cancer risks, suggesting that environmental and lifestyle factors affect breast cancer incidence (Figure 1). In general, Western developed countries have higher incidence rates compared to Asian countries, and Singapore has rates higher than most other parts of Asia. In Singapore, Chinese women are at the highest risk, compared to Malays and Indians.

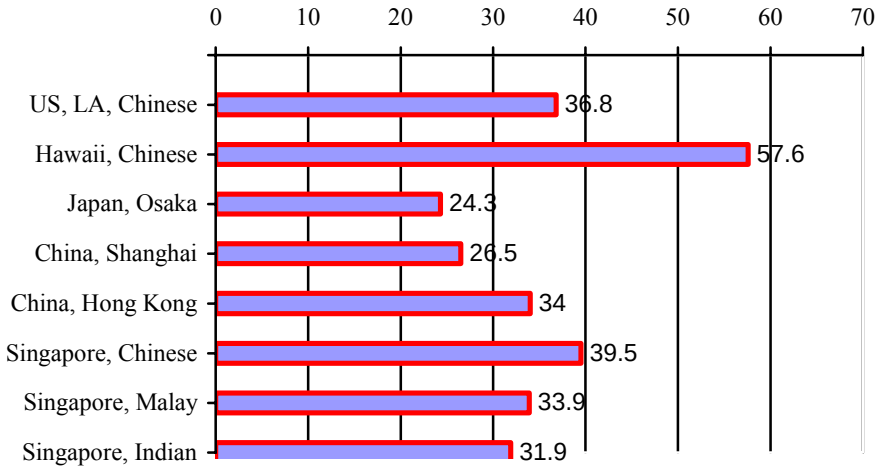


Figure 1. International comparisons of breast cancer incidence - age-standardized rates (per 100 000 per year), 1988-1992 (adapted with permission from Chia *et al.*, 2000), showing differences in breast cancer rates between Chinese people in different countries and between ethnic groups in the same country, reflecting the complex interaction between genetic heritage and environmental influences.

Hereditary factors affect the risk of developing breast cancer. A woman's risk of breast cancer is doubled if she has a first degree relative (mother, sister, or daughter) who developed the disease before the age of 50, and the younger the relative when she developed breast cancer, the greater the risk. Several inherited genetic alterations associated with increased risk of breast cancer are well known. Li-Fraumani syndrome is caused by germline mutations in the tumour suppressor gene p53. BRCA-1 and BRCA-2 are two other tumour suppressor genes in which mutations give increased chance of breast and ovarian cancer, and carriers have a lifetime breast cancer risk of 40 to 85% (Blackwood *et al.*, 1998). The ataxia-telangiectasia gene causes radiation sensitivity in the heterozygous state and increases risk of breast cancer from screening mammography (Lippman, 1998). However, not all familial clusters of breast cancers have defined germline mutations: the search for other breast-cancer-susceptibility genes, especially low-penetrance polymorphisms, is on-going (Meijers-Heijboer *et al.*, 2002).

Breast cancer is also a hormone-dependent disease. There is a dose-dependant relationship between female sex hormone exposure and breast cancer risk. Four events in a woman's life that determine hormonal exposure, affect breast cancer risk:

(1) menarche – each year delay in menarche decreases breast cancer risk by 5% (Hunter *et al.*, 1997);

(2) childbearing – women who have their first full-term pregnancy before 20 years have 30% the risk and women with 5 or more children have half the risk of breast cancer, compared with nulliparous women (Ewertz *et al.*, 1990);

(3) breastfeeding – women who breastfed 25 months or more in their lifetime have 33% decrease in risk of breast cancer compared to those who never breastfed (Layde *et al.*,

1989). A recent meta-analysis revealed that the relative risk for breast cancer decreases by 4.3% for every 12 months of breastfeeding in addition to a decrease of 7.0% from each birth; and the size of decline was no different in developed and developing countries (Collaborative Group on Hormonal Factors in Breast Cancer, 2002); and

(4) menopause – breast cancer risk increases by 3% for each year older at menopause (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). In fact, the larger the number of menstrual cycles during a woman's lifetime, the higher the risk for breast cancer (Clairel-Chapelon *et al.*, 2002). Interestingly, there is also a positive correlation between post-menopausal estradiol levels and breast cancer risk (Key *et al.*, 1999).

Exogenous estrogen exposure also adds to breast cancer risk. Use of hormone replacement therapy in healthy post-menopausal women over a 5 year period will cause an increase of 3.2 cases per 1000 users of age 50-59 years and 4 per 1000 aged 60-69 years (Berai *et al.* 2002). The increase in risk is most pronounced in the continuous combined hormone replacement regimen, in which progesterone and estrogen are given continuously (Weiss *et al.*, 2002). Combined oral contraceptives increase breast cancer risk of current users by 25% (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). This excess risk for both hormonal therapy falls after cessation of use, such that 10 or more years after use stops, no significant increase in risk is evident.

In contrast, use of tamoxifen, an estrogen receptor antagonist, reduces the incidence of breast cancer by 38% in healthy women with high risk of developing the disease (Cuzick *et al.*, 2003).

Other non-hormonal iatrogenic sources of breast cancer risk have also been identified. Exposure to ionizing radiation, especially in women less than 40 years of age, increases the relative risk of breast cancer to between 1.1 and 2.7 if the exposure exceeds 1 Gy; it is estimated that about 1% of breast cancers in the USA is attributable to diagnostic radiology (Evans *et al.*, 1986). Breast cancer incidence increases by about 75 times in women who were successfully treated for Hodgkin's disease, compared to the general population. This effect is attributed to thoracic irradiation and possibly, chemotherapy (Deniz *et al.*, 2003).

Other factors that are associated with increased breast cancer risk include:

(1) presence of proliferative benign breast disease, especially with atypia - four fold increase risk compared to women without benign breast disease (Bodian, 1993);

(2) radiodense breast tissue (Boyd *et al.*, 1998);

(3) alcohol intake - 10% risk increase for every 10g of alcohol consumed per day (Smith-Warner *et al.*, 1998);

(4) obesity in post-menopausal women - 50% increased risk for women with BMI more than equal to 30kg/m^2 compared to lean women (Hunter *et al.*, 1993); and

(5) low level of physical activity (Friedenreich *et al.*, 1998).

Interestingly, possible dietary modulators of cancer risk, such as fat content, meat, fibre, fruit and vegetable, and phyto-estrogen were not consistently associated with breast cancer risk. Exposure to carcinogens, such as smoking and environmental estrogens (e.g. the insecticide DDT), was not found to significantly affect breast cancer incidence (Key *et al.*, 2002).

Clearly, a complex interaction between genetic, physiologic, lifestyle and environmental forces affects the risk of breast cancer in women.

1.3 Anatomy and physiology of the breast

The human breast is one of a pair of accessory reproductive glands that lies on the anterior surface of the chest wall. Its base stretches from the 2nd to the 6th rib along the mid-clavicular line, with the medial two thirds lying on the pectoralis major and lateral one third on the serratus anterior and external oblique, and an axillary tail extending laterally upwards into the axilla. Superficially, it is covered by skin, with a pigmented region, the areola, surrounding the nipple.

Nerves to the breast are derived from the anterior and lateral cutaneous branches of the 4th, 5th and 6th thoracic nerve. Blood supply is from the lateral pectoral and acromiothoracic branches of the axillary artery, the intercostal arteries, and the internal mammary artery, forming an anastomosing network. Venous blood returns through the deep veins that run with the main arteries. Some drainage through the posterior intercostal vein enters the vertebral veins – a pathway for metastatic spread to the spine.

The lymphatic drainage is of great importance with regards to the spread of breast cancer. Lymph vessels that drain the lateral part of the breast pass to the pectoral group of axillary lymph nodes, whereas those from the medial part of the breast perforate the thoracic wall, ending in the internal mammary lymph node. Occasionally, lymphatics from the superior part of the breast drain into the infraclavicular lymph nodes. When the

usual channel of lymph drainage is obstructed by malignant cells especially in advanced tumours, there can be lymph flow between the medial and lateral part of the breast, across to the opposite side of the body, to the supraclavicular lymph nodes, and to the cervical lymph nodes.

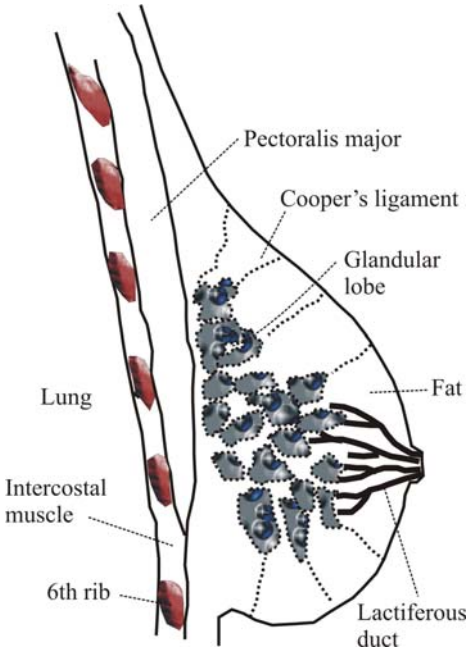


Figure 2. Schematic diagram of sagittal section of a female breast.

The breast parenchyma is made up of fibrous, fatty and glandular tissue. The fibrous strands, known as the Cooper's ligaments, attach the breast to the chest wall. A layer of fat surrounds the breast glands and occupies the space between the glands. The glandular tissue is organized into 15 to 20 lobes, each lobe containing numerous branches of lactiferous ducts. These ducts unite to form larger ducts, ending in one of 15 to 20 excretory ducts that converge towards the nipple, dilating to form sinuses at the base of

the nipple, before opening individually at the tip of the nipple (Figure 2). It was once thought that ductal breast cancers originate from the ducts, whereas lobular breast cancers arise from the lobules. However, it is now known that both arise from the terminal duct lobular unit (Sainsbury *et al.*, 1994). Near the base of the nipple, on the surface of the areola, are numerous sebaceous glands (Montgomery glands) that enlarge during lactation and appear as small tubercles.

The development of the breast is unlike most mammalian organs: a linear developmental phase is followed by a cyclical phase. The mammary epithelium is derived from the ectoderm. At about 5 weeks of gestation, the epidermis forms 2 lines of thickened epithelium (mammary ridges) running cranio-caudally on the ventral aspect of the embryo. The mammary epithelium thickens to form a lens-shaped disc that grows to form a mammary bud. The mammary bud grows into the surrounding mesenchyme and begins to branch to form the rudimentary gland of the neonate. The surrounding dermis differentiates to form the stroma by 32 weeks. The lactiferous ducts emerge at the mammary pit – a depression that evaginates near birth to form the nipple.

Initially, the breast structure appears to develop independently of steroid hormones. After the fifteenth week, testosterone plays an important role. Estrogen and progesterone receptors only appear at about the 30th week of gestation (Keeling *et al.*, 2000). The gland remains in a quiescent state until puberty when ovarian hormones, estrogen and progesterone, stimulate the growth of the terminal end bud. Ducts elongate and cells differentiate into luminal epithelial cells and myoepithelial cells. This continues until the limits of the fat pad is reached, after which duct elongation ceases. It has been suggested

that the duct epithelium is most susceptible to carcinogens (e.g. from smoking) during this period (Russo *et al.*, 1982).

The cyclical phase of breast development begins once pregnancy ensues. Estrogen stimulates the proliferation of lobuloalveolar progenitor cells within the ducts resulting in alveolar formation. Initially, the alveolus is made up of a mass of granular polyhedral cells. Under the influence of prolactin, an anterior pituitary gland hormone whose secretion increases after 8 weeks of pregnancy and rises throughout, they acquire the capacity to produce milk proteins, but secretory function is inhibited.

At parturition, the inhibitory effect of estrogen and progesterone on lactation is lifted by the delivery of the placenta, causing a drop of their levels in the blood. Suckling inhibits the secretion of prolactin inhibitory hormone from the hypopituitary, causing a more rapid rise in prolactin secretion. The central alveolar cells undergo fatty degeneration and are passed out as colostrum, whilst the peripheral cells form oil-laden secretory vacuoles, the contents of which are emptied into the alveolar lumen by exocytosis. Myoepithelial cells that surround the alveoli, contract to express milk upon stimulation by oxytocin, secreted by the posterior pituitary in response to suckling. On weaning, milk secretion stops and the alveolar cells undergo apoptosis; and the rest of the epithelial cells remodels into a ductal gland morphology, awaiting the next pregnancy. It is thought that differentiation of the lobuloalveolar progenitor cells during pregnancy reduces the number of cells susceptible to malignant change, thereby lowering the long-term breast cancer risk of the multi-parous woman (Russo *et al*., 1999).

1.4 Histopathology

Because of the different cell types in the breast, several rare malignancies occur in the breast, including cystosarcoma phyllodes, angiosarcoma, primary lymphoma. These are not epithelial malignancies and are therefore not considered typical breast cancers and will not be further discussed. The bulk of breast cancers is of epithelial origin and present with distinctive histology under the light microscope.

The American Joint Committee on Cancer (AJCC) classifies the histological types into 5 main groups: (1) carcinoma, NOS (not otherwise specified); (2) ductal; (3) lobular; (4) nipple (Paget's disease); and (5) others (undifferentiated carcinoma). Each group is further divided into subgroups, for example, the ductal group of breast cancers, consists of intraductal (in situ), invasive with predominant intraductal component, invasive (NOS), comedo, inflammatory, medullary with lymphocytic infiltrate, mucinous (colloid), papillary, scirrhous, tubular and "other" cancers (AJCC, 2002). In Singapore, like the rest of the world, the invasive ductal breast cancer is by far the most common type of breast cancer (79% of all cases in Singapore from 1993 to 1997), followed by invasive lobular cancer (4.4%) (Chia *et al.*, 2000).

Biological characteristics differ between the histological types. Papillary and mucinous carcinomas tend to occur in older patients compared to those with other types of carcinoma. On the other hand, medullary carcinomas occur in the relatively young, and they have poorly differentiated histology, lymphocytic infiltration and absence of hormone receptors. Lobular cancer tends to have contra-lateral recurrence (Broet *et al.*,

1995). Histological types associated with favourable prognosis include tubular (>97% 5-year disease free survival), mucinous (84%), medullary (78%) carcinomas.

Breast cancers are also classified according to tumour grade – an estimate of differentiation by light microscopy. It is significantly related to frequency of recurrence and death, disease-free interval and overall length of survival after treatment, regardless of clinical stage (Yoshimoto *et al.*, 1993). Histologic grading of invasive ductal breast cancer is commonly based on the Bloom and Richardson criteria: (a) the extent of tubule formation, (b) nuclear pleomorphism, and (c) mitotic rate, are assigned a score of 1 to 3. The total score determines the final grade with well-differentiated (grade I) scoring 3 to 5, intermediate (grade II) scoring 6 to 7 and poorly differentiated (grade III) cancers scoring 8 to 9 (Figure 3).

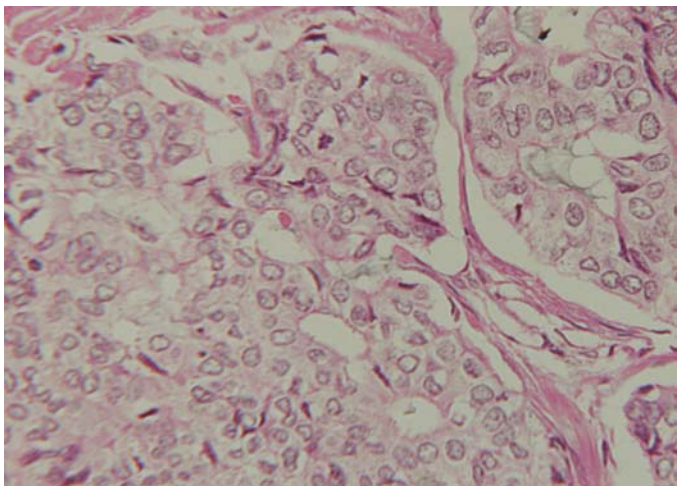
Other microscopic features that may be useful in predicting poor prognosis include:

(1) necrosis – presence of which reflects rapid tumour growth;

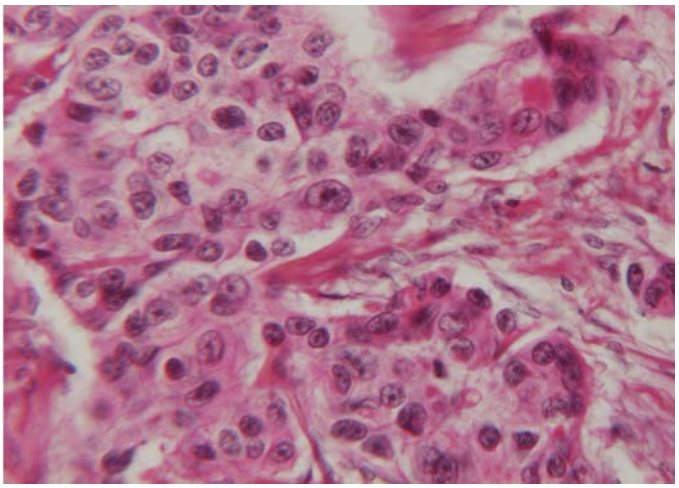
(2) lymphoplasmacytic infiltrate – often associated with medullary carcinoma which has favorable prognosis, however, its occurrence in non-medullary ductal carcinomas are associated with poor differentiation and hormone receptor negativity;

(3) vascular invasion – penetration of lymphatic and blood vessels by tumour cells detected microscopically predicted higher frequency of local recurrence and lower survival rate; lymphatic tumour emboli, in particular, was unfavourable especially in node negative patients, with a 3 times increased risk of recurrence if found in a T1N0M0 cancer;

(4) angiogenesis – high tumoral micro-vessel density has shown promise as a negative prognostic marker, but a standardized method of assessing vascular density has not been established;



(A)



(B)

Figure 3. (A) Grade 1 invasive ductal breast cancer showing well formed tubules with lining cells exhibiting minimal nuclear pleomorphism; **(B)** Grade 3 invasive ductal breast cancer with increased nuclear size and pleomorphism (magnification 160x).

The role of stromal characteristics and extent of intraductal carcinoma in prognostication remains controversial (Rosen, 2001).

Other than cellular and structural characteristics under light microscopy, the expression of certain molecular markers in breast cancers is also of histological interest. Immunohistochemical detection of estrogen receptors (ER) and progesterone receptors

(PR) is used to determine the possibility of benefit of hormonal therapeutic agents and in the risk stratification of node negative breast cancer patients. More recently, tumour proliferative (S-phase) fraction (Hutchins *et al.*, 1999) and level of HER2/neu expression (Thor *et al.*, 1998) are used to guide the selection of adjuvant chemotherapy treatment. The advent of a humanized monoclonal antibody that binds HER2/neu receptor (Herceptin) for treatment of metastatic breast cancers makes HER2/neu immunohistochemistry useful in predicting response to treatment (Burstein *et al.*, 2001).

Molecules that have been extensively studied as immunohistochemical markers, but presently have not found widespread clinical use in breast cancers include MUC 1 (a glycosylated mucin protein), peptide growth factors and their receptor (eg. EGF, EGFR, TGF-α, TGF-β), other oncogenes (eg. ras, c-myc), p53 tumour suppressor gene, cell proliferation markers (eg. Ki67, PCNA) and other molecules (eg. metalloproteases, intermediate filament proteins, basement membrane components, CEA, cathepsin D).

1.5 Staging and treatment

Staging is based on a TNM classification (AJCC, 2002). Tumour size (T), regional lymph node involvement (N) and presence of distant metastasis (M) are used in the staging system. Stage grouping is used to predict survival and as a guide for treatment.

For ductal carcinoma-in-situ, treatment is with total mastectomy, or breast-conserving surgery and radiation therapy. On the other hand, lobular carcinoma-in-situ

may be observed after diagnostic biopsy. In both cancer types, tamoxifen may be added to reduce risk of subsequent cancer progression.

Invasive breast cancers are treated more aggressively. Treatment modalities are chosen bearing in mind the side-effects of treatment and the risk of recurrent disease after treatment.

For operable breast cancers, initial loco-regional treatment may be either modified radical mastectomy, or breast-conserving surgery (lumpectomy) and axillary clearance with breast irradiation.

Adjuvant radiotherapy to the chest wall and lymph node is advised for selected patients with higher risk for loco-regional recurrence after mastectomy, such as those with primary tumours more than 5cm in diameter or having more than 4 lymph nodes, or extra-nodal involvement (Fowble *et al.*, 1988).

Systemic treatment includes cytotoxic chemotherapy and hormone antagonists. For ER or PR positive tumours, tamoxifen (an estrogen receptor antagonist) may be given. A recent trial showed improvements in disease-free survival if letrazole, an aromatase inhibitor, were given after about 5 years of treatment with tamoxifen (Goss *et al.*, 2003), but more detailed analyses of possible bone, lipid, and cardiovascular side effects with the new regime is pending. Medical ovarian ablation using a gonadotropin-releasing hormone analogue in place of, or in combination with tamoxifen is still the subject of clinical evaluation. Popular adjuvant chemotherapy regimes for breast cancer are either anthracycline-based or cyclophosphamide/methotrexate/5-fluorouracil (CMF) based. They are usually given to patients with lymph node positive, or intermediate to high risk node negative tumours, unless age or physical condition does not permit.

The treatment of inoperable, metastatic and recurrent breast cancer will depend on the clinical situation. Often, it involves combined modality treatment, with the goals of improving quality of life and prolongation of life (National Cancer Institute, 2002).

1.6 Apoptosis in breast cancer

The development of cancer is a multi-step process. Aided by genomic instability, the cell undergoes a succession of genetic changes. Those that confer the cell a survival advantage, will be selected by a "Darwinian" process and propagated as the cell progresses from a pre-malignant state to cancer. It is believed that, no matter which genes are modified, virtually all cancers eventually manifest six "hallmark" capabilities: (1) self-sufficiency in growth signals; (2) insensitivity to anti-growth signals; (3) evasion of apoptosis; (4) limitless replicative potential; (5) sustained angiogenesis; and (6) tissue invasion and metastasis (Hanahan *et al*., 2000). Acquisition of the "hallmark capabilities" sets the scene for tumour expansion.

Apoptosis is a process of physiologically programmed cell death manifested by cell shrinkage, formation of blebs, breakdown of nucleus, and fragmentation of DNA and chromatin condensation.

By exploiting its characteristic physical and biochemical changes, apoptosis can be readily detected in tissues. Electron microscopy is able to detail the ultrastructural changes and surface blebbing of apoptotic cells. The characteristic DNA fragmentation can be shown using agarose gel electrophoresis or ELISA: the internucleosomal cleavage

of DNA during apoptosis results in a typical "ladder" fragmentation pattern after DNA electrophoresis; and the nucleosomal complex of histone proteins and double-stranded DNA (dsDNA) are selectively identified with ELISA using an antibody to a histone protein and another to dsDNA. The terminal deoxynucleotidyl transferase-mediated, dUTP-biotin nick end-labeling (TUNEL) method amplifies and detects the binding of dUTP-biotin to terminal 3'-hydroxyl ends of single or double stranded DNA exposed during DNA breakdown. Flow cytometric determination of apoptosis can be performed using propidium iodide and FITC-labelled annexin V simultaneously. Propidium iodide binds to DNA and enters necrotic cells but is excluded from apoptotic cells whilst annexin V binds to phosphatidylserine, which translocates to the outer leaflet of the plasma membrane early in apoptosis (Allen et al., 1997).

Figure 4 illustrates the many possible pathways that lead to apoptosis. Because of the multitude of pathways triggering this self-destructive mechanism, the prevention of cell death is a complicated process.

At the most proximal level, a group of proteins, the IAPs (inhibitor of apoptosis proteins), bind and inhibit the effector caspases, and may also promote their degradation through ubiquitination. During apoptosis, the inhibitory effect of IAPs is lifted by the release of an IAP-binding protein, SMAC (second mitochondria-derived activator of caspase), from the mitochondria. Of the nine IAP family members, XIAP, cIAP1, cIAP2 and survivin have been found to be involved in apoptotic regulation in breast cancer cells. Survivin is over-expressed in 60% to 70% of breast cancers and is associated with reduced apoptotic index in these tumours (Tanaka *et al.*, 2000).

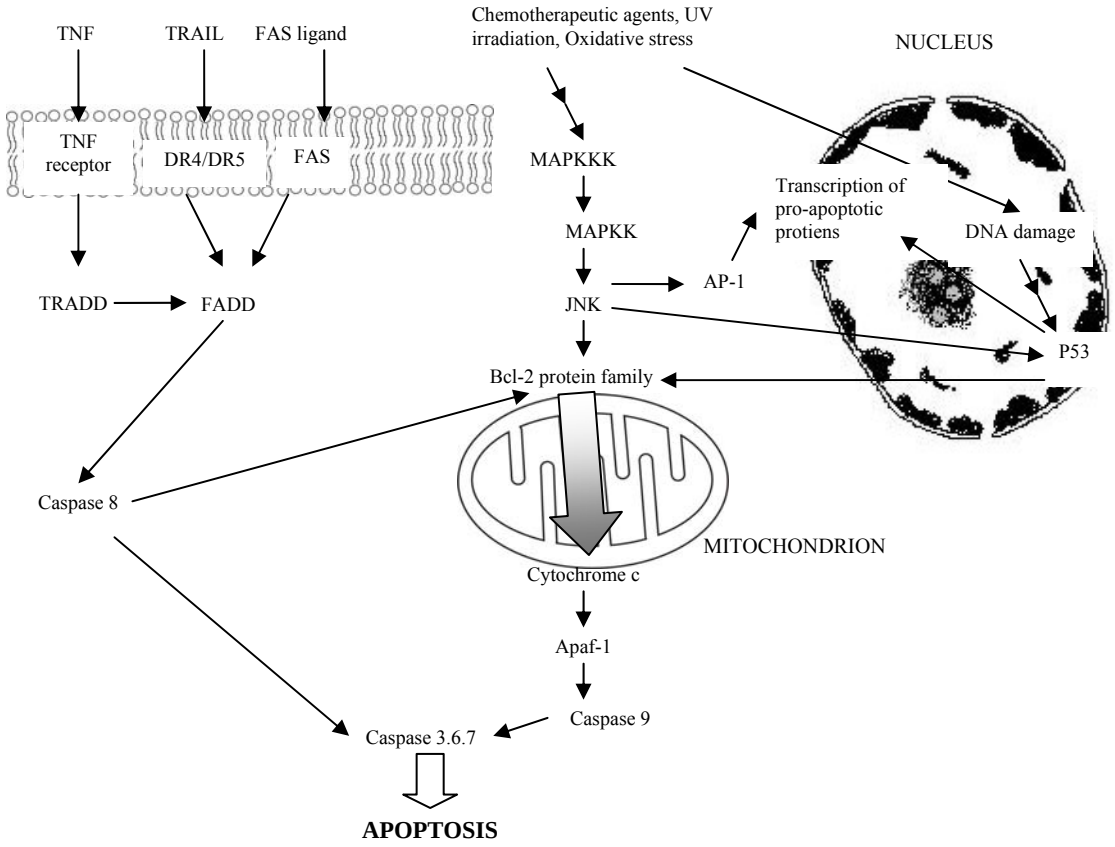


Figure 4. Some of the many pathways to apoptosis. Two major routes are evident: one through activation of death receptors, such as TNF, TRAIL and Fas ligand; another through the mitochondria. These two routes may interact through caspase 8. (MAPKK = mitogen activated kinase kinase; MAPKKK = MAPKK kinase)

Further upstream of the effector caspases, a plethora of anti-apoptotic and pro-apoptotic proteins serve to regulate apoptosis tightly.

Over-expression of anti-apoptotic proteins is a common feature of breast cancer. For example, Bcl-2 and Bcl-xL, when up-regulated, confer resistance to apoptosis in breast cancer. Although both proteins inhibit apoptosis primarily through the mitochondria mediated pathway, over-expression of either protein was shown to be

operative in the resistance of breast cancer cells to apoptosis after activation of the death-receptor apoptotic pathway (Srinivasan *et al.*, 1998 and Fulda *et al.*, 2002), as well as when p53 is up-regulated (Schott *et al.*, 1995). Treatment of a breast cancer cell line with anti-sense oligonucleotide to Bcl-xL induces cytochrome c release and mitochondrial membrane permeability change (Simoes-Wust *et al.*, 2000) and inhibiting Bcl-2 expression in a similar way also promotes apoptosis in breast cancer cells (Chi et al., 2000).

On the other hand, down regulation or mutation of pro-apoptotic molecules confer apoptotic resistance. Pro-apoptotic members of the Bcl-2 family include Bax, Bak, Bad, Bid and Bim. Of these, Bax has been most actively studied in breast cancer patients (Jager *et al.*, 2002). Bax may be mutated, resulting in loss of function in breast cancer (Seitz *et al*., 2003). In transgenic mouse models, partial loss of Bax expression resulted in accelerated progression of mammary tumours (Shibata *et al.*, 1999), whilst induction of Bax expression restored sensitivity to apoptosis and reduced breast tumour growth (Bargou *et al.*, 1996).

Amplification of c-myc is a common occurrence in breast carcinogenesis, but it promotes programmed cell death through the adaptor protein, Bin1. This protein is expressed in normal breast epithelium, and is frequently missing or inactivated in malignant breast cancer cells. In most cases, the down-regulation is attributed to epigenetic causes (Ge *et al.*, 2000), suggesting the importance of suppressing pro-apoptotic factors during tumour growth in breast cancer.

P53 is another pro-apoptotic protein of interest in breast cancer. In addition to causing cell cycle arrest, p53 induces transcriptional activation of pro-apoptotic genes

such as that of Bax, Apaf-1 and DR5. More recently, it is suggested that p53 also binds directly to Bcl-xL and Bcl-2, liberating Bax and Bak from inhibition, thereby causing changes in the mitochondrial membrane that leads to the caspase cascade towards apoptosis (Manfredi, 2003). Inactivating mutations of p53 has been found to worsen prognosis in human breast cancers (Liu *et al.*, 2002).

In many tumours, death receptors are the target of modification to reduce their sensitivity to apoptotic signals. The death receptors may be downregulated, inactivated, or mutated. Breast cancer cells are frequently resistant to Fas mediated apoptosis. The expression of Fas is down-regulated in more breast cancers compared to benign tumours (Mottolese *et al.*, 2000). However, somatic mutation in Fas could not be found in a sample of 48 breast cancer cases (Muschen *et al.*, 2001). Functionally significant mutations of DR4 and DR5 are found only in metastatic breast cancers, suggesting that these genetic adaptations occur late in breast carcinogenesis (Puiu *et al.*, 2003).

Some malignancies secrete decoy molecules that bind death-receptor ligands without initiating any apoptotic signaling cascade. Soluble Fas and DcR3 (decoy receptor 3) compete with trans-membrane Fas for binding with the Fas ligand (FasL). Soluble Fas is transcribed in breast cancers and can be detected in the serum of breast cancer patients. Higher levels of soluble Fas are associated with breast cancers of higher TMN stage (Sheen-Chen *et al.*, 2003). DcR3 may play a lesser role in breast cancers. Serum levels of DcR3 in breast cancer patients are not significantly higher compared to healthy patients, in contrast with that of patients with cancers of gastrointestinal origin (Wu *et al.*, 2003).

1.6.1 Resisting intrinsic pressures for apoptosis

Apoptotic restraint in breast cancer is further enhanced by molecular adaptations to the "sensory" signaling pathways that are set off by environmental and cellular apoptotic pressures. The cellular impetus for apoptosis may arise from the disturbances to such cellular processes as growth and DNA repair.

Deregulated cell proliferation often results in increased apoptotic pressures. Proliferative oncogene, Ras can be detected immunohistochemically in about 60% of breast cancers (Gohring *et al.*, 1999). It acts synergistically with E2F1 (E2 promoter binding factor 1) to activate ARF expression, which in turn stabilizes p53, thereby sensitizing the cell to apoptosis (Berkovich *et al.*, 2003). E2F1 also acts as a counterpoise to the loss of function of tumour suppressor Rb. Rb regulates the G1 cell cycle checkpoint, and the loss of which results in cell proliferation and E2F1-mediated apoptosis (Ginsberg, 2002). In addition to inducing apoptosis through the ARF/Mdm2/p53 pathway, alternative pathways of apoptotic induction may be possible at the same time as E2F1 also upregulates transcription of p73, Apaf-1, pro-apoptotic members of the Bcl-2 protein family, and caspases 3 and 7. Whilst functional loss of E2F1 is not documented in human breast cancers, it is possible that inactivation of other essential pro-apoptotic proteins or increased expression of anti-apoptotic proteins will be required for breast cancer development in the presence of such proliferative stimuli.

Cells defective in DNA repair accumulate DNA mutations, and those defective in apoptosis survive with the mutations, and proliferate. Polymorphisms of one of several proteins involved in DNA repair, such as MLH1, MSH2, ATM, BRCA1 or BRCA2,

occur in some sporadic breast cancers and are frequently associated with familial cancers. These proteins involved in DNA repair, also play a role in initiating apoptosis in the presence of excess DNA damage (Rich *et al.*, 2000). As an illustration, BRCA1 acts in a multi-protein BRCA1-associated genome surveillance complex to sense DNA damage and participate in homologous recombinational repair of double stranded breaks. It also acts as a transcriptional activator for the gene coding for GADD45, which is known to trigger apoptosis through JNK (c-Jun N-terminal kinase). Alternatively, BRCA1 may act through H-Ras proto-oncogene to involve the same JNK pathway (Thangaraju *et al.*, 2000). As a result, carriers of BRCA1 mutations are at a substantially higher risk of developing breast cancer in their life-time compared to the general population. Evasion of apoptosis in the face of DNA damage leads to propagation of mutation and the resulting chromosomal aberrations can be the cause of genomic instability promoting further malignant change.

Further, cells are sensitive to external environmental conditions for "survival signals". Signaling molecules such as cytokines, hormones, growth factors and adhesion molecules, bind to cellular receptors that activate downstream signaling pathways. A lack of these signals usually triggers apoptosis via the PI3K/PDK1/Akt pathway, but the molecular pathway may be modified in breast cancers to inhibit the initiation of the apoptotic process.

Normally, the binding of integrins to the extra-cellular matrix is communicated to the PI3K/PDK1/Akt pathway via integrin-linked kinases, focal adhesion kinases or Shc, resulting in cell survival and proliferation (Grossmann, 2002). Breast epithelial cells will undergo a special form of cell death (anoikis) when cell anchorage is inadequate. This

process is disrupted in breast cancers, facilitating detachment from matrix, hence tumour growth and metastasis (Streuli *et al.*, 1999).

In addition to the regulation of growth and proliferation, growth factor receptors have the ability to trigger/prevent apoptosis. The epidermal growth factor receptors (EGFR), particularly HER2 (or EGFR-2, encoded by gene c-erbB2), plays an important role in breast cancer development. On binding with EGF, the EGFR oligomerizes and exhibits tyrosine kinase activity. Through adaptor protein, Shc, signaling cascades down the Raf/MEK/ERK and PI3K/PDK1/Akt pathways are initiated. Akt phosphorylates and inactivates pro-apoptotic proteins Bad and caspase 9, as well as transcription factors (eg. Forkhead factors) which are responsible for expression of pro-apoptotic genes. The PI3K/PDK1/Akt pathway is negatively regulated by PTEN. Female patients with Cowden syndrome, a disease associated with germline PTEN mutation, have an increased (25-50%) risk of developing breast cancer during their lifetime compared to the general population. The breast cancer may also exhibit EGF independent survival and elude apoptosis by gene amplification of c-erbB2, a phenomenon found in about 25% of breast cancers (Navolanic *et al.*, 2003).

1.6.2 Resisting extrinsic pressures for apoptosis

Even when cells manage to circumvent the intrinsic tendencies to undergo apoptosis, the immune system serves as a watchdog against cancer formation. Immunosurveillance against transformed cells are mainly performed by NK cells and T cells, by inducing apoptosis (Henkart *et al.*, 1997). Histological sections often reveal lymphocytes

surrounding nests of breast cancer cells; and T cell response against breast cancer specific tumour associated antigens, eg. Her2 and MUC1, can be detected in breast cancer patients but not in normal controls (Nagorsen *et al.*, 2003). In order to grow to such clinically significant size in the face of such immune response, the cancer cells will have developed resistance to such targeted cell killing, or develop mechanisms to avoid immune detection.

Lymphocytes secrete perforin and proteolytic enzymes, known as granzymes towards target cells. Perforin causes an increase in plasma membrane permeability, allowing the granzymes to penetrate the target cell cytoplasm. Granzymes, being serine proteases, may cause cell death by cleaving and activating caspases and pro-apoptotic Bid (Barry *et al.*, 2002). Breast cell lines has been shown to express serine protease inhibitor PI-9/SPI-6, which inhibits granzyme B, resulting in immune escape (Medema *et al.*, 2001). It is not known if human breast tumours behave in the same way.

The lymphocytes may also express FasL, which binds to Fas on the cancer cell surface, initiating the death-receptor apoptotic pathway. As described in the previous section, many breast cancers have developed mechanisms to elude Fas activation. In reverse, tumours may over-express FasL to cause Fas-dependent apoptosis in susceptible lymphocytes. This was demonstrated when breast cancer, hepatoblastoma and colonic adenocarcinoma cells caused destruction of co-cultured Fas-positive Jurkat lymphocytes (Muschen *et al.*, 2000). Higher transcription of FasL relative to Fas in breast cancers is associated with poorer prognosis (Reimer *et al.*, 2000). Interestingly, tamoxifen binding to estrogen receptors on breast cancer cells inhibits FasL expression, possibly explaining the protective effect of tamoxifen against breast cancer (Mor *et al*., 2000).

1.6.3 Chemotherapy resistance

Therapeutic induction of apoptosis is another stress that breast cancers have to contend with. Measurable increases in apoptosis occur in breast tumours within 24 hours of starting chemotherapy (Archer *et al.*, 2003). From the 1980s till presently, the use of chemotherapy has become more prevalent and its application is gradually extended to breast cancers of lower risk groups than before (Mariotto *et al.*, 2002). Cytotoxic drugs in common use for adjuvant treatment of breast cancer patients employ a variety of mechanisms to disturb cell processes. They include DNA-damaging agents such as cyclophosphamide and anthracyclines (eg. doxorubicin), anti-metabolites such as methotrexate and 5-fluorouracil, as well as, mitotic inhibitors such as taxanes (eg. docetaxel and paclitaxel).

Exposing breast cancer cells to agents that affect nucleic acid metabolism results in p53 induction through the activation of DNA-repair pathways. Doxorubicin, methotrexate and 5-fluorouracil have all been shown to induce Fas by a transcriptionally regulated p53 mechanism, rendering the cell sensitive to FasL-mediated apoptosis (Muller *et al.*, 1998). At the same time, the stress activated protein kinase, JNK, pathway signaling appears to influence doxorubicin-induced cell cycle withdrawal and control of apoptosis (Kim *et al.*, 2003). On the other hand, micro-tubule active drugs had no effect on p53 expression in breast cancer, but brought about Bcl-2 hyperphosphorylation and inactivation as well as reduced Bax-Bcl-2 dimerization, resulting in apoptosis (Srivastava *et al.*, 1998).

It is not surprising, therefore, that many apoptosis-related markers, such as bcl-2, bax, bcl-xL, bag-1, fas, and fasL have been investigated for their ability to confer chemotherapy resistance in breast cancers (Sjostrom *et al.*, 2002). Inhibition of pro-apoptotic proteins, such as Bim (Sunters *et al.*, 2003) resulted in resistance against chemotherapy agents, whilst inhibition of anti-apoptotic markers, such as Bcl-2 and Bcl-xL increased sensitivity. In fact, the inhibition of these proteins is being studied actively as a potential adjunct treatment to increase sensitivity of breast cancer to chemotherapy (Simoes-Wust *et al.*, 2002).

The over-activation of growth factor receptors, such as from the over-expression of HER2, results in broad spectrum chemo-resistance against both groups of cytotoxic drugs (Knuefermann *et al.*, 2003). It was demonstrated that the resultant activation of the PI3K/PDK1/Akt signalling pathway is primarily responsible for abrogating the apoptotic pressures generated by these drugs (Jin *et al.*, 2003), resulting in reduced survival in Her-2 positive breast cancer patients compared to Her-2 negative patients after exposure to chemotherapy (Walker, 2000).

Other survival strategies that may be employed by the cancer cell include mechanisms that reduce intra-cellular drug accumulation (such as by regulating drug influx and efflux, and drug detoxification) and those that limit the damage caused (such as by altering the drug target and by damage repair). For example, resistance to cyclophosphamide is primarily mediated by increased rates of inactivation of active metabolites through conjugation with glutathione or metabolism to its inactive keto and carboxy metabolites. Methotrexate resistance, in contrast, is probably a complex multifactorial phenomenon – impaired transport of methothrexate into cells, increased

expression of the drug target dihydrofolate reductase or production of altered forms with decreased affinity for the drug, as well as decreased ability to synthesize active metabolites have been reported (Chabner *et al.*, 2001).

In addition to specific resistance mechanisms, there is also a group of detoxification enzymes and drug transporters that may confer resistance to a broad spectrum of chemotherapy drugs. Cytochrome P450, aldehyde dehydrogenase (Sreerama *et al.*, 1997), NAD(P)H:quinone oxidoreductase 1 (Begleiter *et al.*, 1997), glutathione S-tranferase (Salinas *et al.*, 1999), metallothionein (Cherian *et al.*, 2003), cytosolic sulfotransferase (Coughtrie, 2003) all represent major detoxification enzyme systems that play a part in xenobiotic metabolism in breast cancers. Membrane transporter proteins, such as p-glycoprotein (Pgp), multidrug resistance protein 1 and breast cancer resistance protein (Allen *et al.*, 2002), that actively remove multiple substrates from the cell cytoplasm, are operative many breast cancers. The clinical importance of these proteins in the protection of breast cancer from cell death during chemotherapeutic treatment is currently an area of active study and debate.

From the above review of the apoptotic pressures faced by the breast cancer cell, the redundancy of apoptotic pathways makes potentially malignant cells vulnerable to apoptosis. The cancer cell is practically under siege (Figure 5). Acquiring critical molecular adaptations allows it to modulate the severity of the onslaught and survive.

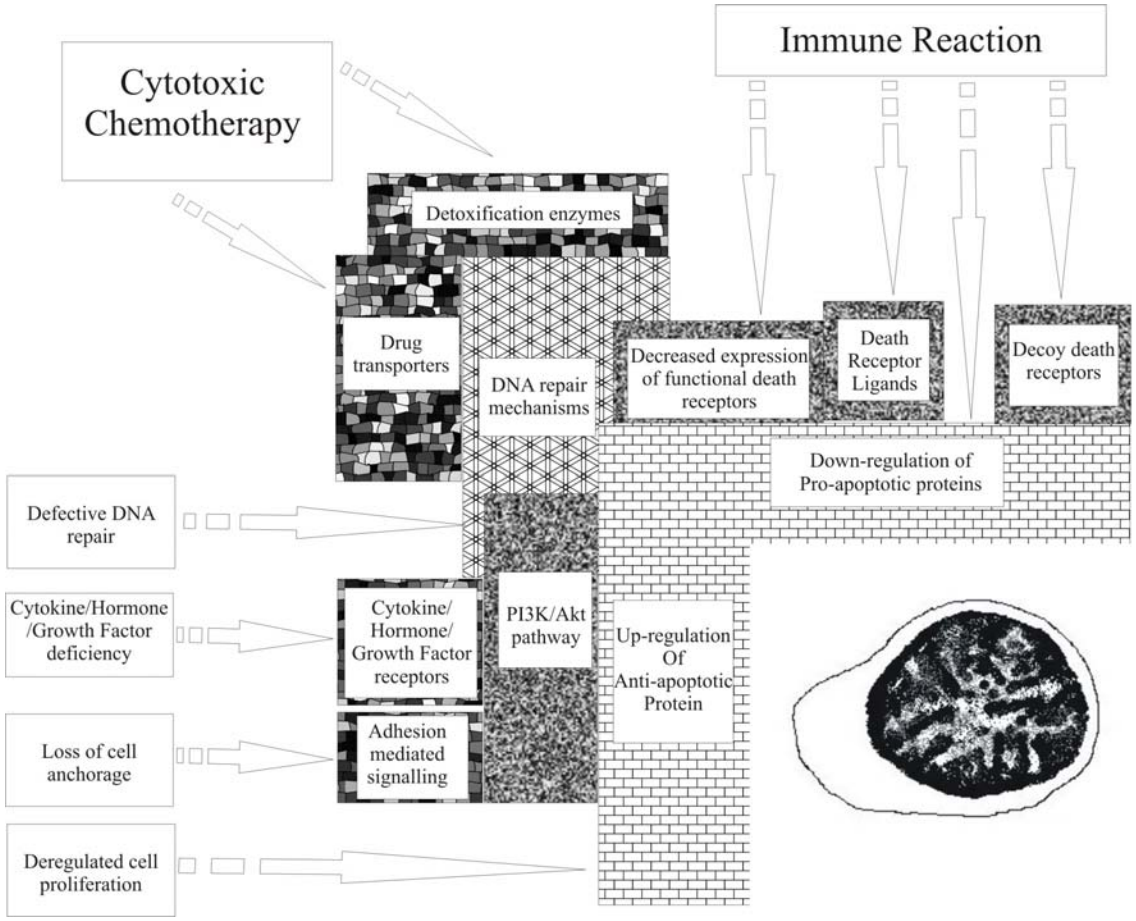


Figure 5. Defence against apoptosis.

1.7 Glutathione S-transferase pi (GST-pi)

Glutathione S-transferases (GSTs) are a family of phase II detoxification enzymes comprising cytosolic and membrane-bound microsomal members. Membrane-bound members trimerize, rather than dimerize to form a single active site, hence constitute a distinct group. Cytosolic GSTs are divided into 6 classes, designated α (alpha), ξ (zeta), θ (theta), μ (mu), π (pi) and ω (omega); the most abundant in mammals being the alpha, mu

and pi classes. They have probably risen from single ancestors, and through mutation, gene duplication and recombination, resulted in broad substrate specificity.

The primary function of GST is to catalyze the conjugation of glutathione to a variety of electrophilic compounds. Substrates include potentially toxic substances such as carcinogens (eg. aflatoxin B, benzo[a]pyrene), pesticides (eg. DDT, parathion), products of oxidative damage (eg. fatty acid hydroperoxides, cholesterol-5,6-oxide, nucleic acid base propenals) and cytotoxic chemotherapy (eg. cyclophosphamide, melphalan). In general, conjugation to glutathione inactivates the parent compound, makes hydrophobic substrates more soluble and facilitates excretion through efflux pumps.

Some members of the GST family, such as GST-pi, have glutathione peroxidase activity (Batist *et al.*, 1986) and catalyze the reduction of organic hydroperoxides (fatty acid, phospholipids, and DNA hydroperoxides) to their corresponding alcohols.

Another interesting feature of GSTs is the ability to act as "ligandin" by binding (sometimes covalently) to various compounds that are not substrates for enzymatic activity, such as steroid and thyroid hormones, bile acids and bilirubin (Litowsky, 1993). This may perform a regulatory function by sequestering the molecules to form an intra-cellular pool or serve a protective function by preventing toxic compounds from interacting with target molecules.

Because of their importance in inactivating potential carcinogens, cytosolic GST expression has been studied as a possible marker that may modify breast cancer risk. Large studies (with more than 400 patients) studying the breast cancer risk of subjects with GST-mu and GST-theta null genotypes have consistently shown no significant increase in risk compared to that of their GST-mu or GST-theta expressing counterparts

(Maugard *et al.*, 1998; Garcia-Closas *et al.*, 1999; and Gudmundsdottir *et al.*, 2001); and research analyzing the polymorphisms of GST-zeta (Smith *et al.*, 2001) and GST-pi (Maugard *et al.*, 2001 and Gudmundsdottir *et al.*, 2001) could not show any correlation between catalytic activity and breast cancer risk.

Also of interest, is the role of GSTs in cancer progression. In breast cancers, the expression of alpha class of GST in breast cancer is low, whilst the expression of GST-mu is consistent with the presence of germ-line polymorphism of the GST-mu gene, hence not likely a factor regulated to promote the persistence of the disease (Shea *et al.*, 1990). GST-pi expression, on the other hand, is widely variable in breast cancers (Kelley *et al.*, 1994).

GST-pi, originally isolated in the human placenta (Guthenberg *et al.*, 1979), is a 210 amino acid sized anionic protein. It is characteristic for high activity against base propenals and other aldehyde products of oxidative stress (Berhane *et al.*, 1994). Although GST-pi null mice appeared healthy, had no obvious signs of distress or illness, and normally developed organs on histopathological examination (Henderson *et al.*, 1998), the importance of GST-pi in cancer cell survival is reflected by the induction of potent apoptosis when GST-pi activity is suppressed by a specific inhibitor (Asakura *et al.*, 2001). Further, GST-pi is up-regulated in breast cancer cells resistant to doxorubicin (Kim *et al.*, 1991), vincristine (Whelan *et al.*, 1992) and cyclophosphamide (Chen *et al.*, 1995), suggesting a role in chemotherapy resistance.

1.8 Scope of study

Assessing the susceptibility of breast cancer to cell death is important as it reveals the potential of the cancer to become more malignant, to colonize potentially hostile environments in distant sites and to survive cytotoxic chemotherapy. Identifying tumour markers associated with these clinically pertinent biological characteristics will allow oncologists to stratify recurrence risk and provide more specific treatment regimes and directed counseling for patients.

In an earlier study (Huang *et al*., 2000), it was shown that there were differences in nuclear shape and size between GST-pi positive and GST-pi negative breast cancer cells within human breast cancer tissues, suggesting that GST-pi may affect nuclear processes in breast cancer. It was felt that GST-pi might be able to inhibit apoptosis in the breast cancers, allowing the accumulation of potentially lethal mutations and chromosomal changes, thereby causing the changes in nuclear morphology.

Based on the observations of the above study, it was hypothesized that GST-pi expression in breast cancers result in clinically significant effects on the disease history with or without chemotherapy. The possibility that this effect is due to inhibition of apoptosis or concomitant expression of other biological markers is further examined.

As such, the following have been evaluated:

1. expression of GST-pi in breast cancer and its association with common clinico-pathological features;
2. relationship of GST-pi expression with total GST activity, lipid peroxidation and apoptosis;

3. clinical significance of GST-pi expression in terms of recurrence-free survival with or without adjuvant chemotherapy;
4. association of GST-pi expression with Bcl-2 and metallothionein (MT) that may provide the breast cancer a "survival" phenotype; and
5. association of GST-pi expression with Y-box binding protein-1 (YB-1) and p-glycoprotein (Pgp) expression, as well as, assessment of their clinical significance especially in patients who received adjuvant chemotherapy.

Materials and Methods

2.1 Patients

This study included 137 women diagnosed with infiltrative ductal breast carcinomas. These patients had no distant metastasis at the time of diagnosis and underwent mastectomy or lumpectomy without neoadjuvant treatment between 1998 and 1999 in the Singapore General Hospital. The study was approved by the instiutional ethics committee. Data on tumour size and grade, lymph node involvement, estrogen and progesterone receptor status were obtained from histopathological records.

Paraffin embedded breast cancer sections from 116 patients were used in terminal deoxynucleotidyl transferase-mediated, dUTP-biotin nick end-labeling (TUNEL) technique, as well as glutathione S-transferase pi (GST-pi) and Bcl-2 immunohistochemistry.

2.1.1 Clinico-pathological characteristics

The age of the patients ranged from 33 to 86 years, with a median age of 52 years; 66 (57%) of the patients were more than 50 years of age. Twenty-one (18%) of the tumours were stage T1 (≤ 2cm in greatest dimension), 82 (71%) were stage T2 (more than 2cm to 5cm in greatest dimension), whilst the rest of the tumours were stage T3 (more than 5cm in size) or T4 (local extension into chest wall or skin); 11 (9%) were histologically grade I, 43 (37%) were grade II and the majority of the tumours were grade III; 74 (64%) of the primary tumours were estrogen receptor positive, 40 (34%) were estrogen receptor negative whilst estrogen receptor status was not analyzed for 2 patients at the time of

diagnosis; 63 (54%) had lymph node metastasis at diagnosis, 51 (44%) did not whilst 2 other patients did not undergo axillary lymph node dissection.

A more detailed study of the biological associations of GST-pi was conducted in tumour samples from 32 different patients for whom the tissues could be collected in cold 0.9% saline solution immediately after surgery. After macroscopic examination, a section of at least 1 cm^3 was rapidly frozen in liquid nitrogen and stored until further use in the measurement of total glutathione S-transferase (GST) activity and quantitation of thiobarbituric acid reactive substances (TBARS) for each of the tumours. The remaining tissues were fixed in formalin and embedded in paraffin for histological analyses.

Further immunohistochemical studies of Y-box binding protein-1 (YB-1), metallothionein (MT) and p-glycoprotein (Pgp) were performed on a random sample of 99 tumours from the set of 116 tumours. The clinico-pathological characteristics of this sample are comparable with the parent set of tumours (see Table 1).

2.1.2 Patient treatment

Chemotherapy treatment was prescribed for 71 (61%) of the 116 patients. The regimes were noted from the case records and confirmed with attendance records from the National Cancer Centre, Singapore. Thirty-six (51%) patients received the cyclophosphamide/methotrexate/5-fluorouracil (CMF) regime, whereas the rest received doxorubicin-based regimes such as doxorubicin/cyclophosphamide, doxorubicin/taxol, and cyclophosphamide/doxorubicin/5-fluorouracil.

Table 1. Summary of clinico-pathological characteristics

Clinico-pathological factors	Number of tumours		
Total number of tumours in study group	116	99	32
Median Patient age (range)	52 (33-86)	52 (33-86)	55.5 (44-85)
50 years and below	50 (43%)	43 (43%)	10 (31%)
More than 50 years	66	56	22
Primary tumour			
T1	21 (18%)	18 (18%)	7 (22%)
T2	82 (71%)	70 (71%)	21 (66%)
T3 and T4	13	11	4
Regional lymph nodes			
0	51 (44%)	47 (48%)	12 (38%)
1-3	32 (28%)	26 (26%)	10 (31%)
>3	31 (26%)	24 (24%)	10
Unknown	2	2	0
Hormonal receptors			
Positive	74 (64%)	64 (65%)	21 (66%)
Negative	40 (34%)	33 (33%)	11
Unknown	2	2	0
Histologic grade			
I	11 (9%)	10 (10%)	4 (13%)
II	43 (37%)	37 (37%)	13 (41%)
III	62	52	15

2.1.3 Patient follow-up

Patients were followed up for recurrence over durations ranging from 33 to 1464 days with a median follow-up of 1117 days.

2.2 Cell culture

Breast cancer MCF7 (American Type Culture Collection Catalogue Number HTB-22) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 5% fetal calf serum, 2 mmol/L glutamine, 100 units/ml penicillin and 100 micrograms/ml streptomycin. Cells were grown in a humidified atmosphere of 5% carbon dioxide at 37°C.

HeLa cervical cells (American Type Culture Collection Number CCL-2) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37ºC.

Chicken DT40 cells were cultured in a 5% CO2 incubator in growth medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 1% chicken serum and 0.1mM 2-mercaptoethanol) at 39.5ºC.

2.3 Immunohistochemistry

Sections were cut from paraffin-embedded tissue at a thickness of 4µm and mounted on APES (3-aminopropyl-tri-ethoxysilane, Sigma-3648) slides. They were then prepared for immunohistochemistry by dewaxing in 2 changes of xylene and rehydrating to water through a series of alcohol of decreasing concentration. Details of subsequent steps for the detection of each protein are elaborated individually below.

2.3.1 Bcl-2

Sections were pre-heated in 10mM citrate buffer at pH 6.0 before incubation with primary antibody for antigen retrieval. Buffer solution, phosphate buffered saline (pH 7.4) solution was used to remove excess reagents in between each step. The sections were immersed in 0.1% hydrogen peroxide for 30min and 4% normal horse serum for 1h to block endogenous peroxidase and non-specific antibody binding, respectively. A mouse monoclonal to human Bcl-2 (Cymbus Biotechnology) was applied at 1:20 dilution for 90 min. After washing, and incubation with the appropriate secondary antibodies for 1h, avidin-biotin-peroxidase complex was applied for 1 hr at room temperature to amplify the specific binding of primary antibody. Visualization was achieved by incubating with 3,3'-diaminobenzidine tetrachloride (Sigma) as the peroxidase substrate. The sections were then counterstained with hematoxylin. Buffer solution was substituted for the primary antibody in the negative control. Normal ductal epithelium on the same slide was used as internal positive controls as Bcl-2 is known to be expressed in the epithelium of normal breast tissue, ductal hyperplasia and atypical ductal hyperplasia (Siziopikou *et al.*, 1996).

2.3.2 Glutathione S-transferase pi (GST-pi)

Without pre-treatment for antigen retrieval, endogenous peroxidase was blocked by 0.1% hydrogen peroxide in methanol for 30 min, followed by blocking of non-specific binding of antibodies in 5% normal goat serum for 1h, before sections were incubated at room

temperature with primary antibody (Dako, USA) at 1:200 dilution for 2h. Conditions were optimised to ensure minimal stromal staining. For negative control, buffer (Tris buffer pH 7.4) was substituted for the primary antibody.

Secondary antibody biotinylated anti-rabbit immunoglobulin (Dako, 1:200 dilution) was then applied for 30 min, followed by a 30-min incubation with biotin and avidin-peroxidase complex (Dako). The immunostaining was demonstrated using diaminobenzidine and hydrogen peroxide for 10 min. The sections were counterstained with methyl green.

2.3.3 Metallothionein (MT)

Commercially available murine anti-human monoclonal E9 antibody (Dako) raised against a conserved epitope of MT-1 and MT-2 isoforms was used as the primary antibody. Endogenous peroxidase was blocked by 0.5% hydrogen peroxide in methanol for 15min, followed by blocking of non-specific binding of antibodies in 5% normal horse serum for 1h, before sections were incubated at 4°C overnight. For negative control, buffer (Tris buffer pH7.4) was substituted for the primary antibody.

Secondary antibody biotinylated anti-mouse immunoglobulin (Dako, 1:200 dilution) was then applied for 30 min, followed by a 30-min incubation with biotin and avidin-peroxidase complex (Dako). The immunostaining was demonstrated using diaminobenzidine and hydrogen peroxide for 10 min. The sections were counterstained with methyl green.

2.3.4 P-glycoprotein (Pgp)

P-glycoprotein was detected using a mouse anti-human monoclonal JSB-1 antibody (Chemicon, 250µg/ml) which reacts with a conserved cytoplasmic epitope of the protein. The immunohistochemical method used was optimized from that described in Faneyte *et al.*, 2001. Dewaxed sections were microwave-heated for 25min from cold. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 10min and non-specific antibody binding blocked in 4% normal horse serum for 30min. Primary antibody was then applied at a 1:20 dilution for 1 hour. In the negative control, buffer solution (0.5M Tris-buffered saline pH 7.4) replaced the primary antibody. A normal liver section was used as positive control.

Detection and amplification of primary antibody binding was performed using anti-murine secondary antibodies (Dako) at 1:200 dilution for 30min and a 10-min incubation with biotin and avidin-peroxidase complex (Dako). Visualization was achieved by the usual method described above with hematoxylin used as the counterstain.

2.3.5 Y-box binding protein-1 (YB-1)

Two different anti-YB-1 antibodies (gifts from Dr Ken Matsumoto, Institute of Physical and Chemical Research, Japan) were used for detection of YB-1 expression. They are rabbit polyclonal antibodies separately raised against frog YB-1 protein and a novel chicken N-terminus deleted YB-1 protein (Figure 6), designated Frgyb-1 and Ckyb-1, respectively.

Dewaxed sections were first heated in 10mM citrate buffer at pH 6.0 for antigen retrieval. Endogenous peroxidase was blocked by immersion of slides in 0.3% hydrogen peroxide in methanol for 15min, followed by blocking of non-specific antibody binding, using 5% normal goat serum over 1hr. Frgyb-1 IgG was used at an IgG concentration of 45µl/ml, whilst anti-serum containing Ckyb-1 was used at dilution of 1:200 in buffer solution, 0.5M Tris-buffered saline pH 7.4. They are applied at room temperature overnight. Secondary antibody - biotinylated anti-rabbit immunoglobulin (Dako, 1:200 dilution) - was then applied for 30 min, followed by a 30-min incubation with biotin and avidin-peroxidase complex (Dako). The immunostaining was demonstrated using diaminobenzidine and hydrogen peroxide for 10min. The sections were counterstained with hematoxylin.

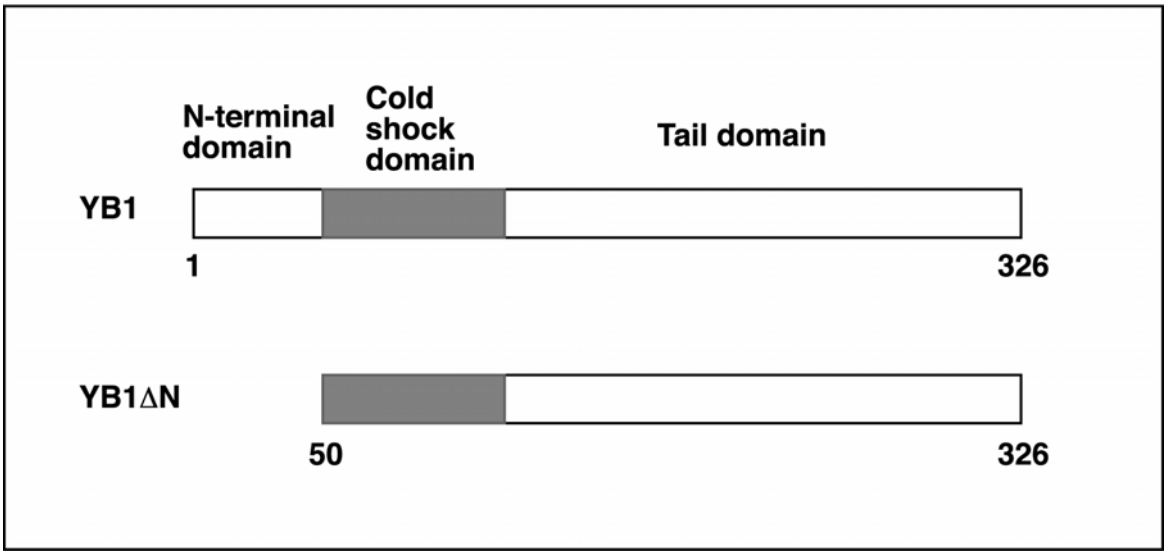


Figure 6. Structure of novel chicken N-terminus deleted YB-1 protein used against which, rabbit polyclonal antibodies (Ckyb-1) are raised.

2.3.5.1 Immunoblotting

MCF7 breast cancer cell line were washed 3 times with ice cold phosphate-buffered saline and lysed with boiling lysis buffer containing 10mM Tris, pH 7.4 and 1% SDS (sodium dodecyl sulfate).

HeLa and chicken DT40 cells were washed with phosphate-buffered saline and lysed by sonication in 5mM Tris HCl pH 7.5, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreitol and 0.25mM phenylmethylsulfonyl fluoride.

The lysates were passed through a 27 gauge needle to shear nucleic acids and boiled for a further 5 min. Lysates were centrifuged at 14,000 *g* for 10 min and the clear supernate transferred to a new tube. Protein estimations were carried out using the BCA kit (Pierce, Rockford Il., USA). 20µg of protein were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Nitrocellulose membranes were blocked for 2 hours with 5% milk in TBST (10mM Tris pH8.0, 100mM NaCl and 1% Tween-20) buffer. Membranes were then separately probed with Frgyb-1 and Ckyb-1 antibodies in 5% milk in TBST overnight at 4°C. The membranes were washed with TBST and incubated with a goat anti-rabbit peroxidase conjugated antibody (Pierce, Rockford Il., USA) for 2 hours at room temperature. Excess antibody was removed by further washes with TBST. The bound antibodies were visualized by chemiluminescense. Membranes were stripped and reprobed with anti-actin antibodies (Chemicon Int. Inc., Temecula, CA, USA).

2.4 Quantification of immunohistochemical staining

The stained slides were viewed using a 40x objective of a light microscope, and 10 random fields were selected and scored.

Bcl-2 expression was classified as positive when more than 30% of tumour cells exhibited cytoplasmic staining, based on levels found previously to be clinically discriminating (Silvestrini *et al*., 1994).

GST-pi expression was considered to be positive when more than 10% of tumours cells exhibited cytoplasmic or nuclear staining (Molina et al., 1993).

Pgp over-expression was classified as positive when cancer cells exhibited cytoplasmic or plasma membrane staining patterns (Filipits *et al.*, 1996).

As MT was expressed in a large proportion of breast cancers (88% in this study), MT immunopositivity is ranked based on an immunoreactive score devised in an earlier study from the same laboratory (Jin *et al.*, 2001). The immunoreactive score is the product of staining intensity (graded 0 to 3) and percentage of immunopositive cells.

Similarly, semi-quantitative determination of YB-1 expression was performed using a different immunoreactive score (ranging from 0 to 15) modified from Janz *et al.* (2002). It is derived from the product of intensity of staining score and percentage score. Intensity of staining was scored from 0 (no detectable immunoreactivity) to 3 (strong immunoreactivity). The percentage of cancer cells expressing YB-1 is divided into 5 groups and scored as such: 1, <20%; 2, 21-40%; 3, 41-60%; 4, 61-80%; 5, 81-100%.

2.5 Immunofluorescence

Co-localization of GST-pi and Bcl-2 were visualized using confocal microscopy after double immunolabelling with fluorescent antibodies. Paraffin embedded, 4μm sections were dewaxed in xylene and rehydrated to water through a series of alcohol of decreasing concentration. They were blocked in 1% bovine serum albumin (BSA) (Sigma) in phosphate buffered saline (PBS) for 1 hour at room temperature. Next, the sections were incubated with anti-Bcl-2 antibody (1:20 dilution) overnight at 4°C. FITC-conjugated goat anti-mouse secondary antibody (to detect the Bcl-2 antibody) at a dilution of 1:200 was then applied for 1 hr at room temperature. The sections were then washed with PBS before incubating with the second primary antibody - anti-GST-pi antibody (1:200) – overnight at 4°C. This was then detected with Cy3-conjugated sheep anti-rabbit secondary antibody (1:800 dilution) for 1 hour at room temperature. In the negative control, BSA was used instead of the primary antibodies. After washing in PBS, the sections were mounted with fluorescence mounting media (DAKO). Stained sections were viewed and photographed using the LSM 510 Carl Zeiss confocal laser scanning microscope (equipped with an argon laser) under a Plan Apo 63 x 1.4 NA (oil) objective. Excitation wavelength for Cy3 was at 543 nm and for FITC at 488nm.

2.6 Detection of apoptosis by TUNEL technique

Apoptosis in tissue sections was identified by the detection of DNA fragmentation using the terminal deoxynucleotidyl transferase-mediated, dUTP-biotin nick end-labeling

(TUNEL) technique with the commercially available TdT-FragELTM DNA Fragmentation Detection kit (Oncogene Research Products, USA). After deparaffinization and rehydration, slides were permeabilized in 20mg/ml of proteinase K in 10mM Tris pH 8 at room temperature for 20 minutes. Endogenous peroxidase was inactivated by treating with 3% hydrogen peroxide. Equilibration buffer is then applied, afterwhich the sections were end-labeled with biotinylated deoxy-neucleotide triphosphate by terminal deoxynucleotidyl transferase for 2 hours at 37°C. The reaction was stopped by a stop buffer. Labeled cells were detected using a steptavidin-horseradish peroxidase conjugate followed by diaminobenzidine staining. The sections were counterstained with methyl green, dehydrated and mounted.

The stained sections were then evaluated by examining cancer cells in 10 random fields of a 40x objective of a light microscope (Zeiss Axioplan). The apoptotic index was defined as the number of apoptotic nuclei per 100 cancer nuclei. 3 tumours were not included in analysis as there was insufficient invasive tumour in the sections for accurate quantitation of apoptotic index.

2.7 Total glutathione S-transferase (GST) activity

Frozen breast cancer tissues were thawed on ice, blotted with filter paper and weighed. They were then homogenized in sufficient 50mM phosphate buffer pH 7.4 solution under standard conditions to make a 10% homogenate. The homogenate was centrifuged at 40 000 rpm at 0 degree Celsius, to obtain a cell-free supernatant. Total GST activity was

determined by measuring the rate of conjugation of glutathione and 1,2-chloro-2,4-dinitrobenzene (CDNB). 10µl cell-free tissue homogenate was added to a mixture of 950µl of 0.1M phosphate buffer pH 6.5, 20µl of 50mM CDNB in ethanol, and 20µl of 50mM glutathione in phosphate buffer. The reaction at ambient temperature of 25°C was monitored by the rise in optical density at 340nm. Correction for non-catalyzed reaction was made by subtracting the rate of change of optical density without enzyme from that with tissue homogenate. One unit of GST activity is defined as the amount of enzyme necessary to conjugate 1nmol of CDNB with 1nmol of glutathione per min.

2.8 Quantitation of lipid peroxidation

Breakdown products of lipid peroxidation react with 2-thiobarbituric acid to form an easily detectable chromogen. Quantifying thiobarbituric acid reactive substances (TBARS) from tissue extract is a standard assay for lipid peroxidation (Ohkawa *et al*, 1979). Briefly, a reaction mixture of total volume 3ml was constituted from 0.2ml of cell-free tissue homogenate, 0.2ml of 8.1% sodium dodecylsulfate, 1.5ml of 1% phosphoric acid, 0.1ml of distilled water and 1ml of 0.6% thiobarbituric acid, was heated for 45min at 100 degrees Celsius. 4.0ml of n-butanol was then added to extract the pink chromogen obtained at room temperature. The fraction dissolved in n-butanol was separated from the rest of the reaction mixture by centrifugation at 1000g for 5min. The optical density of the n-butanol layer was determined at 535nm.

2.9 Computational analysis

The Resonant Recognition Model (RRM) was used for computational analysis of direct interaction between YB-1 and the Y-box region of the MDR1 gene promoter. RRM is based on the theory that protein-protein or protein-DNA interaction depends on the resonant electromagnetic energy transfer at a specific frequency for each interaction (Cosic, 1994). The sequences of seven Y-box proteins (Kloks *et al.*, 2002) were obtained from the National Centre for Biotechnology Information (NCBI) database (Table 2). Three promoter sequences of MDR1 genes were retrieved from the Eukaryotic Promoter Database (EPD ID: EP35012, EP35017 and EP35016).

Table 2. Accession numbers of Y-box proteins (NCBI database)

YB proteins	Accession Number
YB-1 human	P16991
DbpA human	P16989
YB-1 mouse	P27817
EF1 human	AAA30497.1
mRNP3 frog	P45441
mRNP4 frog	P21574
YB-1 frog	P21573

The RRM power spectra were then calculated for each of the sequences. "Consensus" cross-power spectra were calculated for the seven power spectra of the Y-box proteins, as well as for the three spectra of MDR-1 promoter sequences. The two spectra were then analyzed for any common frequency component.

2.10 Statistical analysis

For statistical analysis, SPSS software Windows release 11.5.0 was used. Associations between categorical variables were studied using either Chi-squared or Fisher exact test. The immunoreactive scores of MT, Frgy-1 and Ckyb-1 were treated as non-parametric variables since their distribution were non-normal, and were hence analyzed using non-parametric statistical tests, such as Mann-Whitney test, Wilcoxon signed-ranks test and Spearman rank correlation. The Student t test was used to compare the mean apoptotic index for different groups of breast tumours.

Logistic regression was used for multivariate analysis of tumour factors associated with GST-pi expression. For multivariate analysis of factors associated with apoptosis, log transformation and multiple linear regression was used. Survival curves were plotted by the Kaplan-Meier method and the differences between the curves were evaluated by log-rank test. To assess the factors influencing recurrence, multivariate analysis using Cox's proportional hazard models was performed. A p value of less than 0.05 was considered statistically significant.

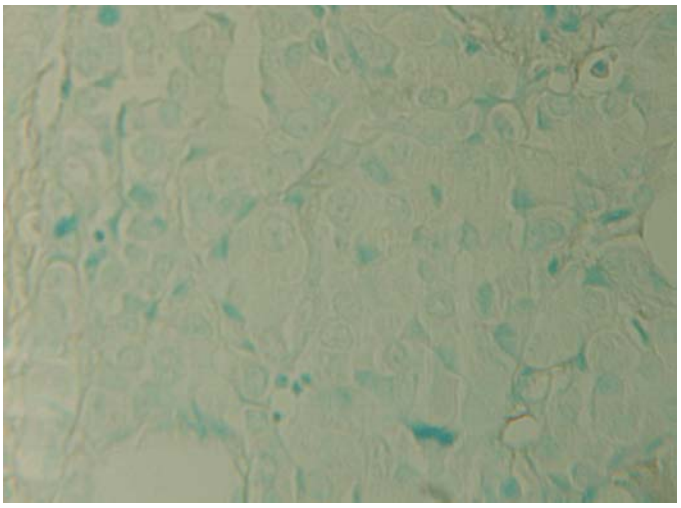
Results

3.1 Glutathione S-transferase pi (GST-pi) expression

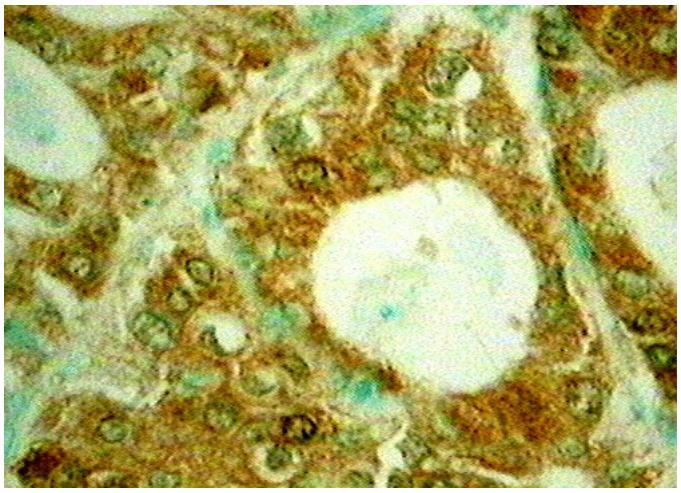
GST-pi expression was found to be present in 58% (67) and negative in 42% (49 cases) of the breast tumours. GST-pi immunostaining was usually observed in the cytoplasm of the breast cancer cells, but was also localized in the nuclei of 15 of the breast tumour samples. Amongst the GST-pi positive tumours, intensity of immunohistochemical staining was variable: 13 (19%) with high expression (Figure 7B), 23 (34%) with moderate expression and 31 (46%) with low expression (Figure 7C). The mean percentage of cancer cells stained in each of the tumours was 45% (standard deviation 32%), with 50% of the GST-pi positive tumours having less than 36% of cancer cells expressing detectable levels of GST-pi.

Peri-tumoral ductal epithelium could be found in 43 of the breast cancer sections. 74% (32) of them had GST-pi positive ductal epithelium (Figure 8). There was no clear association between GST-pi status of the cancer tissue and that of its surrounding normal ductal epithelium ($p = 0.728$). 14 of the sections with GST-pi positive ductal epithelium were found to be associated with GST-pi positive cancers, whilst in the other 18 samples, GST-pi negative cancers were observed in close proximity. In 6 of the 11 sections with GST-pi negative peri-tumoral breast epithelium, the nearby cancer tissues were found to be GST-pi positive (Table 3).

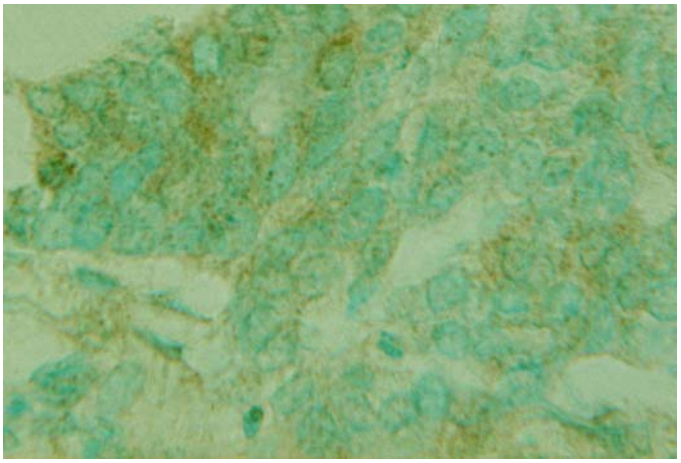
Using the Chi-squared test, GST-pi expression status in the breast cancers was not associated with age, size of primary tumour, histologic grade, estrogen receptor status and the presence of lymph node metastasis (Table 4).



(A)



(B)



(C)

Figure 7. (A) Negative control for GST-pi immunochemistry; **(B)** GST-pi positive breast cancer showing strong diffused cytoplasmic staining in contrast with **(C)** showing a tumour with low GST-pi expression (magnification 250x)

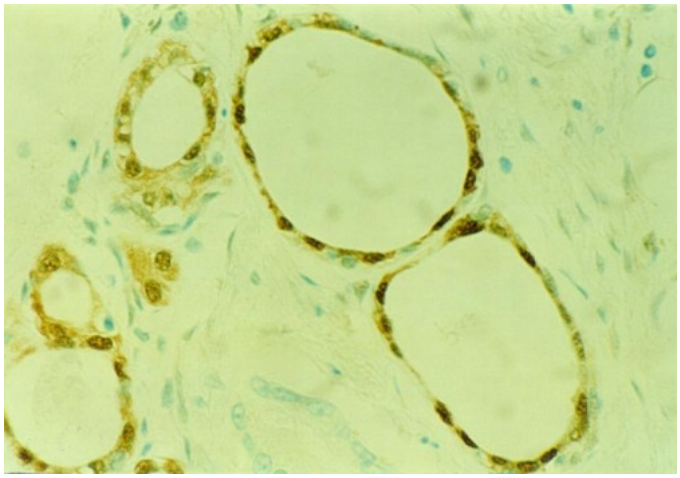


Figure 8. GST-pi expression was detected in the peri-tumoral ductal epithelium (magnification 100x).

Table 3. Lack of association between GST-pi positive breast cancers and GST-pi positivity in their peri-tumoral ductal epithelium.

GST-pi expression status in peri-tumoral normal breast epithelium	GST-pi expression status of breast cancers	
	Number GST-pi positive	Number GST-pi negative
Number GST-pi positive	14	18
Number GST-pi negative	6	5

Table 4. Association between GST-pi expression and clinico-pathological factors

Clinicopathological factors	Number GST-pi positive	Number GST-pi negative	p value
Patient age			
50 years and below	31	19	
More than 50 years	36	30	0.453
Primary tumour			
T1	12	9	
T2	47	35	
T3 and T4	8	5	0.613
Histologic grade			
I	7	4	
II	24	19	
III	36	26	0.894
Estrogen receptor			
Positive	44	30	
Negative	23	17	0.845
Regional Lymph nodes			
Positive	35	28	
Negative	30	21	1.000

3.2 Total Glutathione S-transferase (GST) activity

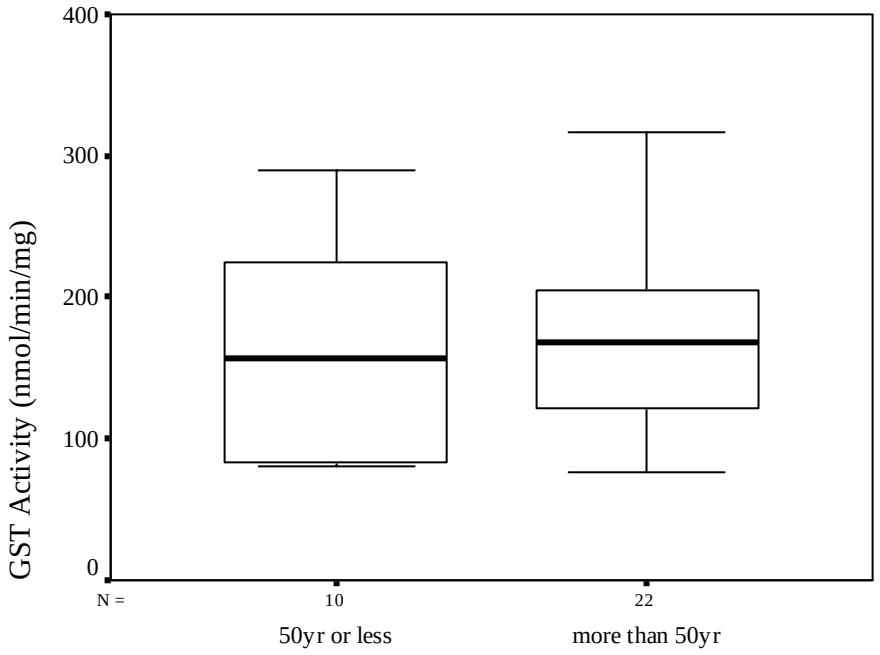
Total GST activity ranged from 76 to 317 nmol/min/mg protein with a median value of 163 nmol/min/mg protein. The activity in GST-pi positive tumours was significantly higher than that of GST-pi negative tumours ($p = 0.041$, Table 5).

As shown in the box-plots in Figure 9, there were no significant differences in GST activity between subgroups of cancers based on age at diagnosis, lymph node status,

hormone receptor status, size and grade of tumour analyzed. The Mann-Whitney test was used to derive the p values due to sample size.

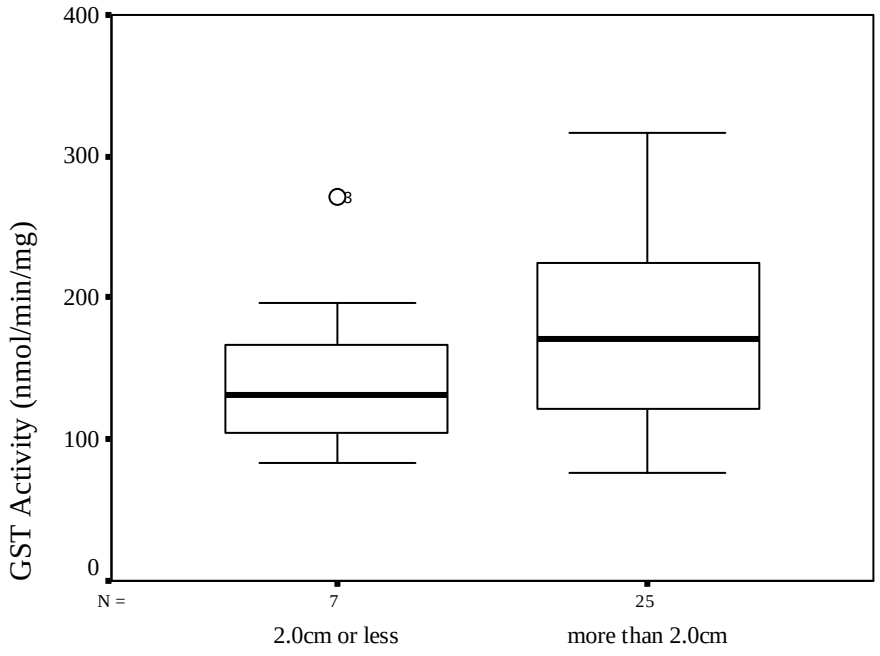
Table 5. Total GST activity in GST-pi positive compared with GST-pi negative cancers

	GST-pi positive cancers (nmol/min/mg protein)	GST-pi negative cancers (nmol/min/mg protein)
Minimum	76	80
25^{th} percentile	138	84
Median	194	131
75^{th} percentile	237	159
Maximum	317	271



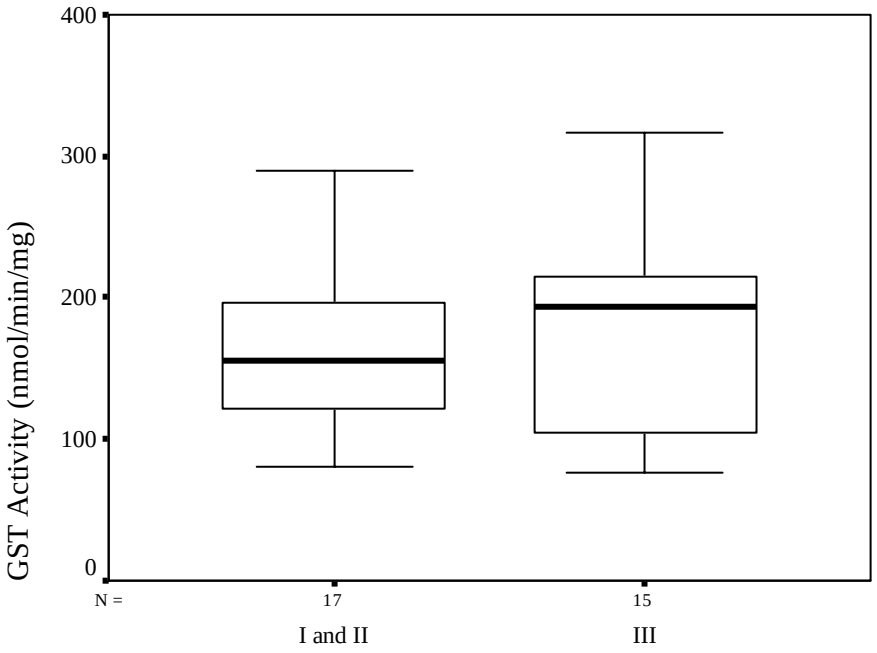
Age (p = 0.62)

(A)



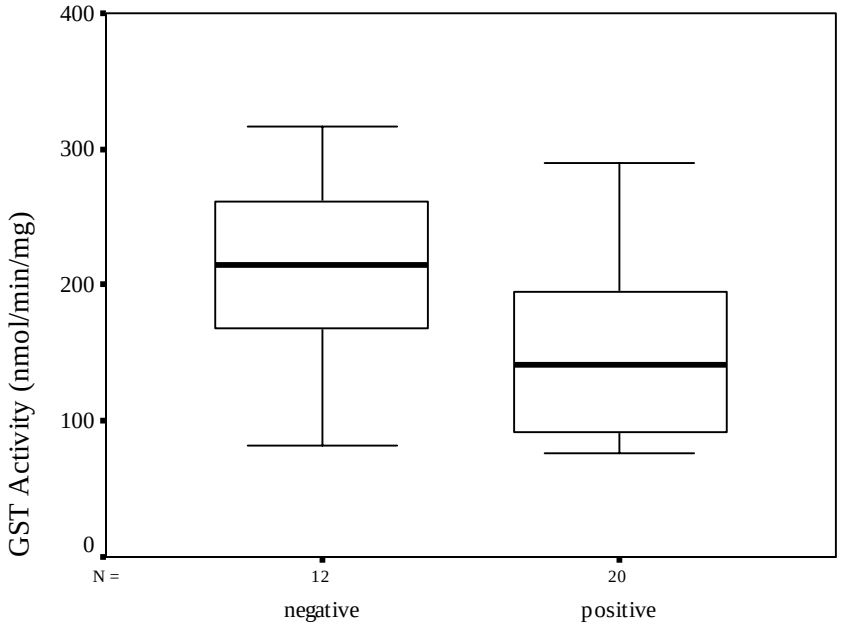
Tumour size (p = 0.33)

(B)



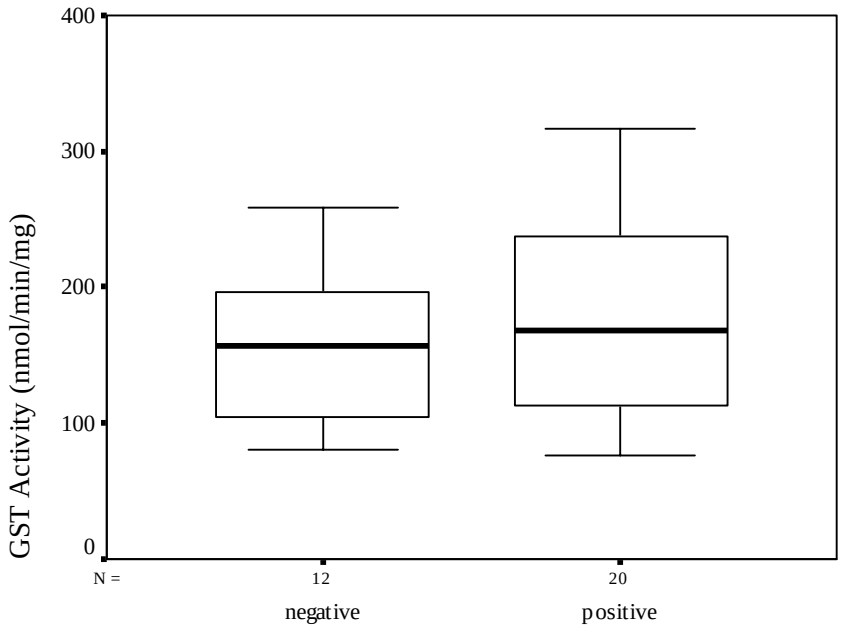
Tumour grade (p = 0.75)

(C)



Estrogen receptor status (p = 0.37)

(D)



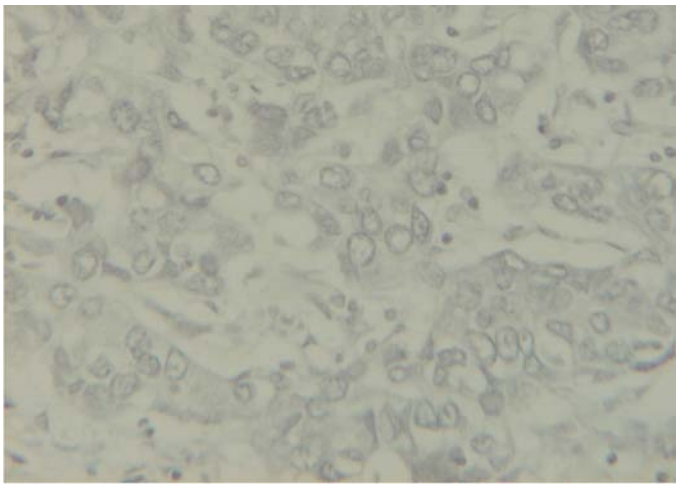
(E)

Figure 9. Box-plots indicating the difference in total GST activity between breast cancers divided into groups based on clinico-pathological characteristics, *viz.* **(A)** age, **(B)** tumour size, **(C)** tumour grade, **(D)** estrogen receptor status and **(E)** lymph node metastasis.

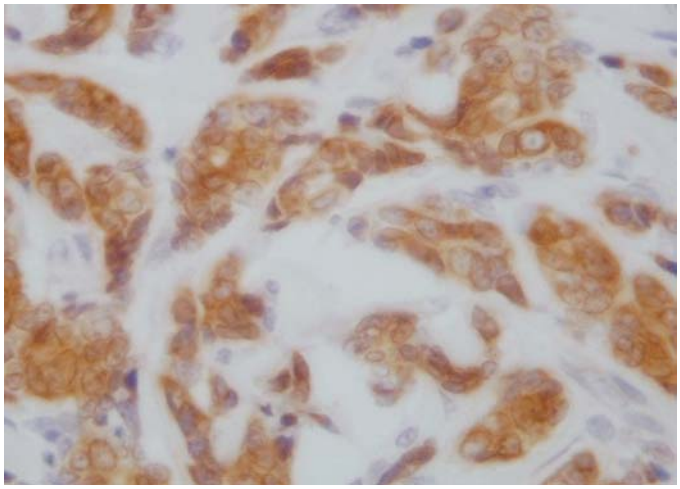
3.3 Bcl-2 expression

Bcl-2 expression was present in 43 (37%) and negative in 72 (63%) of the tumours studied. Immunostaining was localized in the cytoplasm of the cancer cells (Figure 10).

Bcl-2 expressing tumours were generally smaller compared to their Bcl-2 negative counterparts. The mean and standard error of the mean of largest tumour diameter for Bcl-2 positive tumours were 3.02cm and 0.19cm, respectively, whereas that for Bcl-2 negative tumours were 3.85cm and 0.23cm. When compared using the t-test, a statistically significant p value of 0.013 was obtained.



(A)



(B)

Figure 10. (A) Negative control for Bcl-2 immunohistochemistry with primary monoclonal antibody replaced by buffer solution after boiling in citrate buffer for antigen unmasking; **(B)** Bcl-2 positive breast tumour with typical Bcl-2 expression in the cytoplasm of the cancer cell (magnification 100x)

This result was probably not due to outliers in tumour size within both groups of tumours as the proportion of Bcl-2 positive tumours was highest in stage T1 tomours (62%). The proportion of Bcl-2 positive tumours gradually decreased in the groups of stage T2 tumours (33%), and stage T3 and T4 tumours (23%).

Table 6. Association between Bcl-2 expression and clinico-pathological factors

Clinicopathological factors	Number Bcl-2 positive	Number Bcl-2 negative	p value
Patient age			
50 years and below	23	27	
More than 50 years	20	46	0.120
Primary tumour			
T1	13	8	
T2	27	55	
T3 and T4	3	10	0.021
Histologic grade			
I	6	5	
II	16	27	
III	21	41	0.425
Estrogen receptor			
Positive	35	39	
Negative	6	34	0.001
Regional Lymph nodes			
Positive	21	42	
Negative	21	30	0.560

Bcl-2 expression was associated with the expression of estrogen receptor in breast cancers (Table 6). 47% (35 out of 74 tumours) of estrogen receptor positive tumours were Bcl-2 positive as compared to 15% (6 out of 40 tumours) for estrogen receptor negative tumours. This difference in proportion was statistically significant ($p = 0.001$) using the Chi-squared test.

There was no statistical association between Bcl-2 expression and age ($p = 0.120$), histological grade ($p = 0.425$) and presence of lymph node metastasis ($p = 0.560$).

3.4 Association between GST-pi and Bcl-2 expression

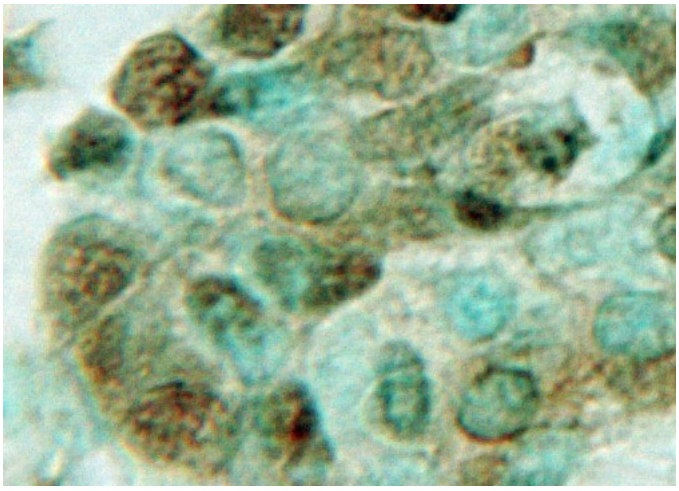
In all, there were 33 GST-pi negative/Bcl-2 negative tumours, 40 GST-pi positive/Bcl-2 negative tumours, 16 GST-pi negative/Bcl-2 positive tumours and 27 GST-pi positive/Bcl-2 positive tumours. There appears to be no association between the expression of both proteins ($p = 0.441$).

However, it was noticed that tumours with nuclear localization of GST-pi were generally Bcl-2 positive. Of the 15 GST-pi positive tumour with nuclear localization, 13 were Bcl-2 positive (Table 7).

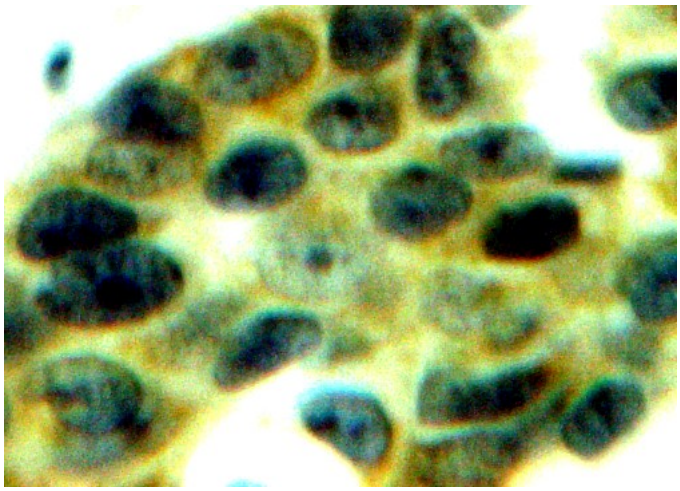
Table 7. Asssociation between breast cancers with GST-pi localization in the nucleus and Bcl-2 expression ($p < 0.001$).

	GST-pi positive breast cancers	
Bcl-2 expression	Number with nuclear localization	Number with only cytoplasmic expression
Number Bcl-2 positive	13	14
Number Bcl-2 negative	2	38

As an example, Figure 11 demonstrates a GST-pi positive tumour with GST-pi immunostaining in the cancer cell nuclei and the presence of Bcl-2 expression in the same tumour.



(A)



(B)

Figure 11. GST-pi positive breast cancer exhibiting nuclear localization of GST-pi detected by immunohistochemistry **(A)** and section from the same tumour showing Bcl-2 immunopositivity **(B).** (magnification 400x)

Bcl-2 expression and nuclear localization of GST-pi in the same cancer cells was demonstrated by double immunofluorescence study. In the tumour section used for Figure 12, GST-pi (red) was expressed in both the cytoplasm and nucleus, whereas Bcl-2 (green) was expressed in the cytoplasm only. The cytoplasmic co-localization of Bcl-2

and GST-pi in the same cells resulted in orange (red + green) fluorescence, and this was associated with red fluorescence (GST-pi detection) in the nuclear regions.

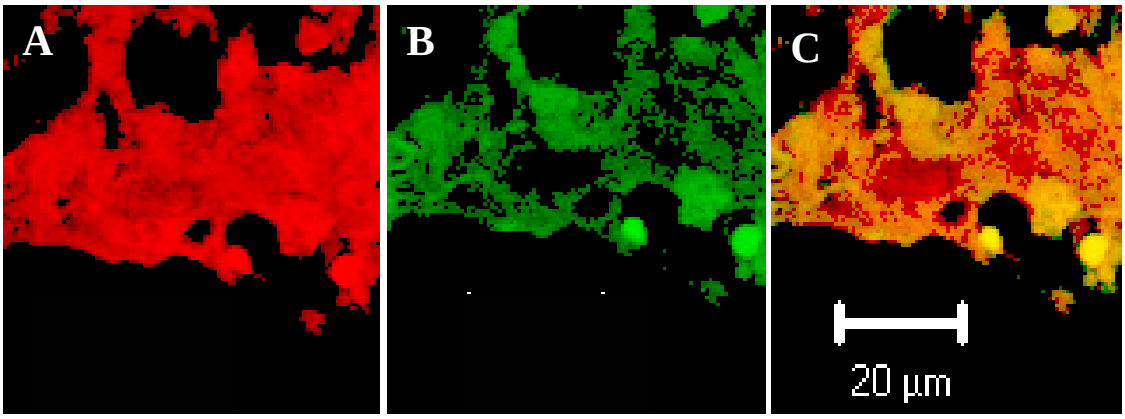


Figure 12. Composite figures of double immunofluorescence staining of GST-pi (**A**, **B,** and **C**). **(A)** Breast cancer cells staining positively with red fluorescence for positive GST-pi immunoreactivity. (**B)** Breast cancer cells staining positively with green fluorescence for positive bcl-2 immunoreactivity. (**C**) Co-localization of GST-pi and bcl-2 in the cytoplasm of the breast cancer cells (orange fluorescence). Nuclear localization of GST-pi is indicated by the red fluorescence. (A, B and C, Bar = 20 µm).

3.5 Y-box binding protein-1 (YB-1) expression

Two different antibodies were used separately to detect YB-1 expression in breast cancer. One was raised against frog YB-1 protein and the other, against a novel chicken N-terminus deleted YB-1 protein. Western blots were performed on human cancer cell lysates to demonstrate the ability of the antibodies to detect human YB-1 (Figure 13 and 14). Both antibodies were able to react specifically to the protein in the lysates.

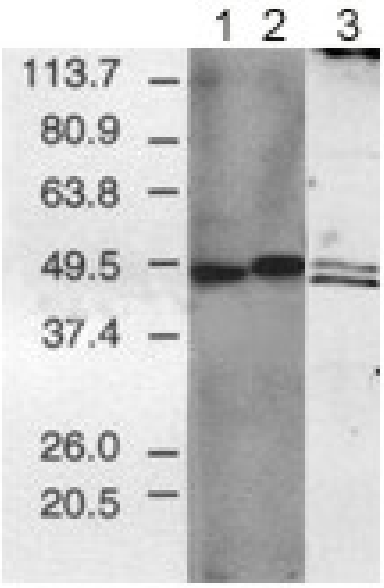


Figure 13 Detection of YB-1 with Ckyb-1 antibody showing single band on Western blot of human (HeLa) cell lysate in lane 1. A similar band is detected in chicken cell lysate in lane 2. Lane 3 shows Western blot of a breast cancer cell line (MCF7) using the same antibody.

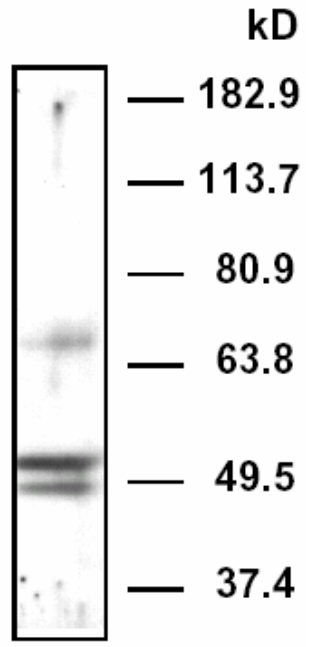


Figure 14. Frgyb-1 antibody detects 2 bands in the region of 49.5 kD in Western blot of HeLa cell lysate.

When Ckyb-1 was used, YB-1 expression was detected in all 99 breast cancer samples, whereas Frgyb-1 demonstrated YB-1 expression in 94 of the breast cancers. The statistical distribution of Frgy-1 and Ckyb-1 immunoreactive scores are shown in Table 8. Although the median scores are the same, Ckyb-1 scores are generally higher than Frgy-1 scores. The Wilcoxon signed-ranks test revealed that Ckyb-1 score was higher than Frgy-1 score in 41% of the tumours and the same score was obtained using either of the antibodies in 37% of tumours ($p = 0.030$).

However, there was significant correlation between immunoreactive scores obtained using Frgy-1 and Ckyb-1. Using simple linear regression, Frgy-1 immunoreactive score was 0.91 times that of Ckyb-1, with 95% confidence interval between 0.77 and 1.04 ($p < 0.001$). Figure 15 is a series of box-plots illustrating how Ckyb-1 immunoreactive score varies for each level of Frgy-1 immunoreactivity.

Table 8. Statistical distribution of Frgy-1 and Ckyb-1 immunoreactive scores

	Frgy-1 immunoreactive score	Ckyb-1 immunoreactive score
Minimum	0	1
25^{th} percentile	1	2
Median	4	4
75^{th} percentile	6	8
Maximum	15	15

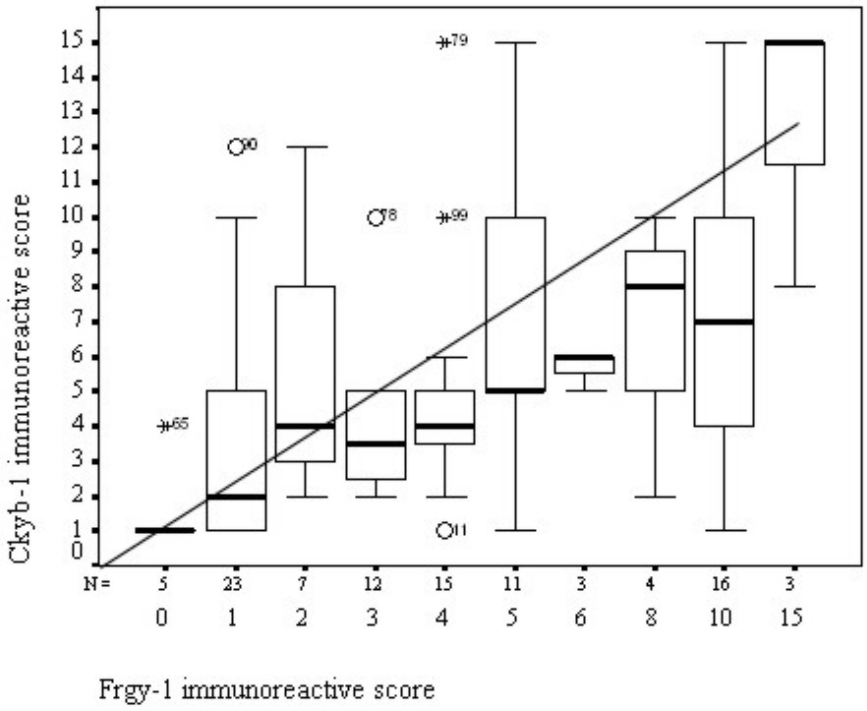


Figure 15. Significant correlation between immunoreactive scores determined using the two antibodies Ckyb-1 and Frgy-1 ($p < 0.001$)

For the purpose of statistical analysis, the immunoreactive scores were classified into 3 groups: low, moderate and high levels of expression. The 25th percentile of the scores was taken as the lower cut-off, whilst the 60th percentile was used as the higher cut-off. For Ckyb-1 immunoreactive scores, immunoreactive scores 0 to 2 were considered low; immunoreactive scores 3 to 5, moderate; immunoreactive scores 6 to 15, high. Since Frgy-1 scores were generally lower, immunoreactive scores 0 to 1 were classified as low; immunoreactive scores 2 to 4 as moderate; and immunoreactive scores 5 to 15 as high.

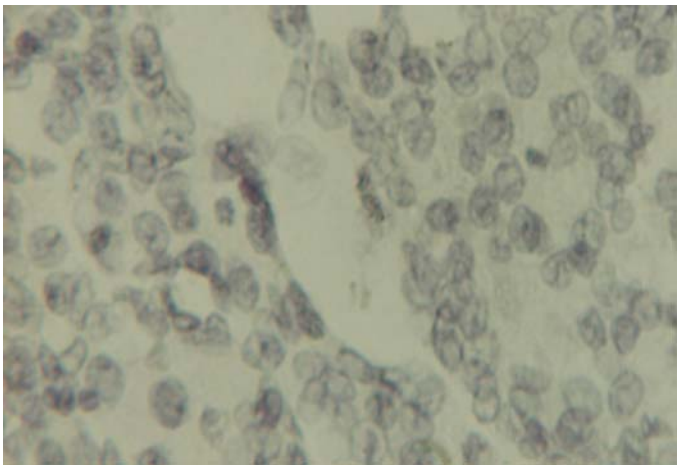
Using this classification, Ckyb-1 classified 27 as having low levels, 40 having moderate levels, and 32 having high levels of expression. Twenty-eight had low levels, 34 had moderate levels, and 37 had high levels of expression with Frgy-1.

YB-1 expression was most commonly observed in the cytoplasm of the cancer cells. Figure 16C and 16D illustrate predominant cytoplasmic localization of YB-1 expression in an estrogen receptor negative breast cancer from a 64 year old patient with no lymph node metastasis. The more intense staining using Ckyb-1 could be discerned in Figure 16D.

Of the 36 samples for which peri-tumoral benign breast tissues were available, 29 (81%) exhibited YB-1 expression. The expression in such tissues was not associated with the immunoreactive score in the corresponding cancer sections.

In a small group of tumours, YB-1 expression may be localized in the nucleus. Figure 16B is an example of such a case from estrogen receptor positive, lymph node negative breast cancer of a 57 year old patient.

Eleven samples exhibited nuclear localization of the protein when antibody Ckyb-1 was used, whilst immunohistochemical detection with Frgy-1 revealed 4 of such tumours. One tumour was common to both groups. Approximately 2-10% of YB-1 positive cancer cells exhibited nuclear expression in each of the samples.



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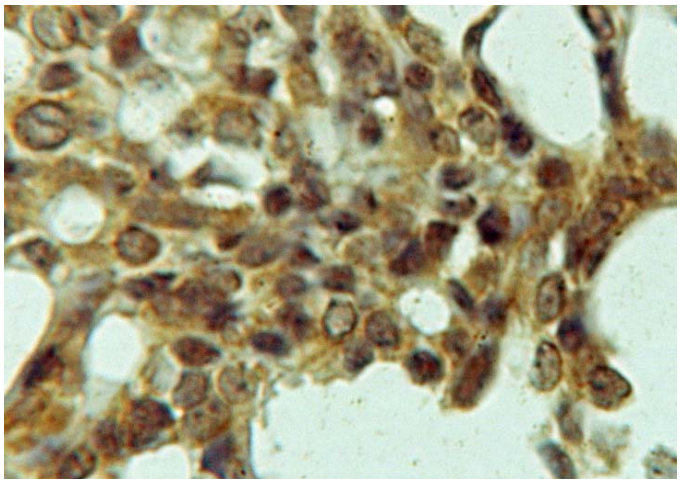

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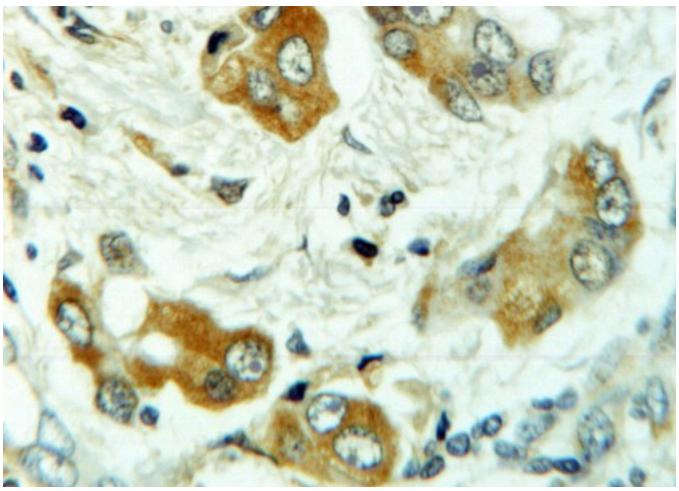

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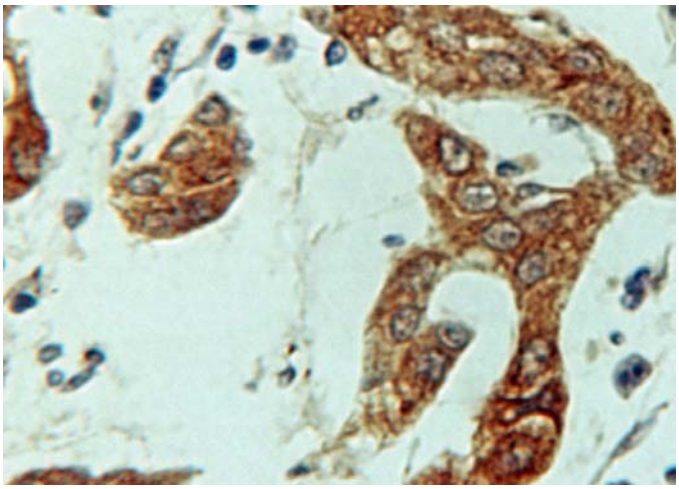


(D)

Figure 16. Detection of YB-1 expression: **(A)** negative control; **(B)** breast cancer section exhibiting nuclear localization of YB-1 detected with Ckyb-1 antibody; **(C)** and **(D)** are sections from the same breast tumour, showing similar expression of YB-1 in the cytoplasm when different antibodies (Frgy-1 or Ckyb-1, respectively) are applied to each section. (magnification 250x)

The level of expression of YB-1 was not associated with individual clinicopathological factors (Table 9). Interestingly, (using Frgy-1 immunoreactive score) it was found that the group of breast cancers of poorest prognostic characteristics (lymph node positive and estrogen receptor negative), has the highest proportion (11 of 18 tumours, or 55%) of breast cancers with high YB-1 expression, compared to that of the group with intermediate prognosis (lymph node positive/estrogen receptor positive or lymph node negative/estrogen receptor negative, 39%) and that of the group with the best prognosis (lymph node negative and estrogen receptor positive, 26%). There is a significant difference in proportion between the extreme groups ($p = 0.035$), as well as a statistically significant trend ($p = 0.037$).

Table 9. YB-1 protein expression levels in different subgroups of breast cancers.

Factors	Ckyb-1 immunoreactive score (median)	Frgyb-1 immunoreactive score (median)
Patient's age		
50 years and below	5	3
More than 50 years	4	4
p value	0.77	0.11
Primary tumour		
T1 and T2	4.5	4
T3 and T4	4	4
p value	0.87	0.75
Regional lymph node metastasis		
Negative	4	3
Positive	5	4
p value	0.11	0.33
Estrogen receptor		
Positive	4	3
Negative	5	5
p value	0.14	0.22
Histological grade		
I and II	5	4
III	4	3
p value	0.42	0.83

Using Ckyb-1 immunoreactive score also revealed the same trend (Table 10). The group of breast cancers with poorest prognostic characteristics (lymph node positive and estrogen receptor negative), had the highest proportion of breast cancers with high YB-1 expression (9 of 18 tumours, or 50%), compared to that of the group with intermediate prognosis (lymph node positive/estrogen receptor positive or lymph node negative/estrogen receptor negative; 16 of 45 tumours, or 36%) and that of the group with

the best prognostic parameters (lymph node negative and estrogen receptor positive; 7 of 32 tumours, or 22%) ($p = 0.017$).

Table 10. Increasing proportion of tumours with high YB-1 expression in breast cancers of poorer prognostic category

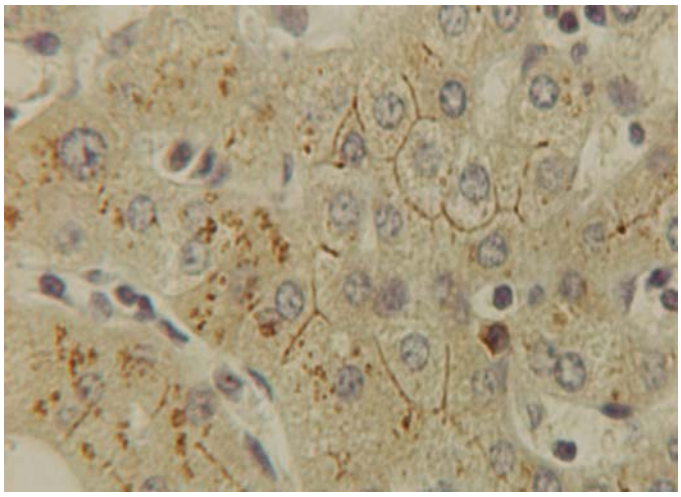
	Prognostic category		
	Ln -/ER +	Ln -/ER - or Ln +/ER+	Ln +/ER -
Number of tumours	32	45	18
Number with high Ckyb-1 score	7 (22%)	16 (36%)	9 (50%)
Number with high Frgy-1 score	8 (25%)	17 (39%)	11 (55%)

Ln = Lymph node; ER = estrogen receptor

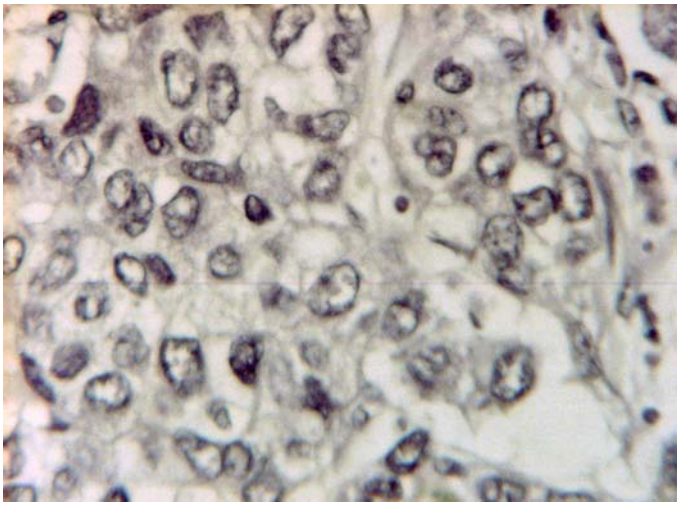
3.6 P-glycoprotein (Pgp) expression

Fifty-seven of the group of 99 breast cancer patients underwent chemotherapy and their Pgp expression was further studied immunohistochemically. 43% (24) of these 57 tumours exhibited Pgp immunopositivity.

Normal liver tissue was used as positive control for the experiment. Figure 17A shows Pgp detected in both the cytoplasmic membrane and cytoplasm of the hepatocytes. A similar pattern of Pgp expression was demonstrable in breast cancer (Figure 17C), although the level of expression (intensity of staining) was generally lower than the liver control.



(A)



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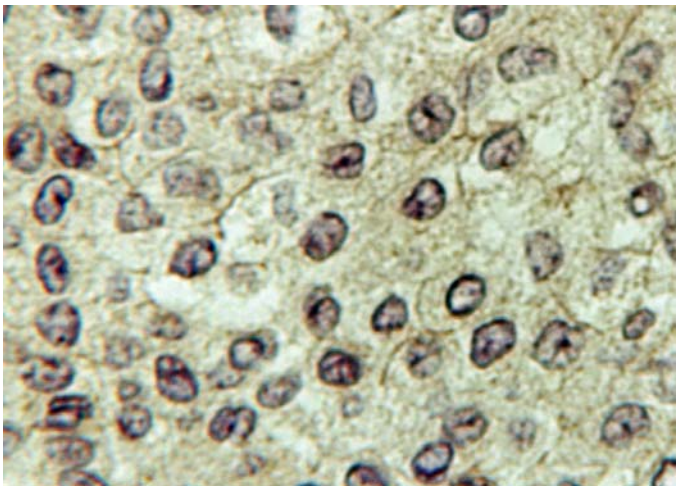


(C)

Figure 17. Sections of **(A)** a normal liver acting as positive control, **(B)** negative control and **(C)** a typical breast cancer section exhibiting cytoplasmic and plasma membrane patterns of Pgp expression. (magnification 250x)

Table 11. Association between Pgp expression and clinico-pathological factors

Clinicopathological factors	Number Pgp positive	Number Pgp negative	p value
Patient age			
50 years and below	19	16	
More than 50 years	5	17	0.028
Primary tumour			
T1 and T2	21	30	
T3 and T4	3	3	0.689
Histologic grade			
I and II	10	18	
III	14	15	0.424
Estrogen receptor			
Positive	19	17	
Negative	5	15	0.044
Regional Lymph nodes			
Positive	14	16	
Negative	9	17	0.560

In this group of patients who received chemotherapy post-operatively, Pgp expression in breast cancer was found to be more common in patients who are 50 years of age and below, compared to those more than 50 years old (Table 11). In the former group of patients, 54% (19) were Pgp positive, whereas only 23% were Pgp positive in the latter group ($p = 0.028$).

Pgp expression appeared to be less common in estrogen receptor negative tumours (25%), compared to their estrogen receptor positive counterparts (53%). This association was statistically significant ($p = 0.044$) by the Chi-squared test. Pgp

expression was not associated with the size of the primary tumour ($p = 0.689$), tumour grade ($p = 0.424$) and the presence of lymph node metastasis ($p = 0.560$).

3.7 Association between YB-1 and Pgp expression

The possible interaction between YB-1 and the Y-box promoter element of the MDR1 gene (coding for Pgp) was explored by computational analysis using the resonant recognition model (RRM). Figure 18 shows the RRM power spectra and cross power spectrum of seven Y-box binding proteins listed in Table 2. The first seven plots are the respective power spectra for each of the proteins. The last plot is the cross power spectra. There is a prominent peak around 0.0215 ± 0.0066, which represents the characteristic frequency. According to the RRM theory, it may assumed that 0.0215 ± 0.0066 is the characteristic frequency representing the binding between YB-1 and Y-box sequence, since the major common feature among the seven Y-box proteins is their ability to bind the Y-box DNA sequence.

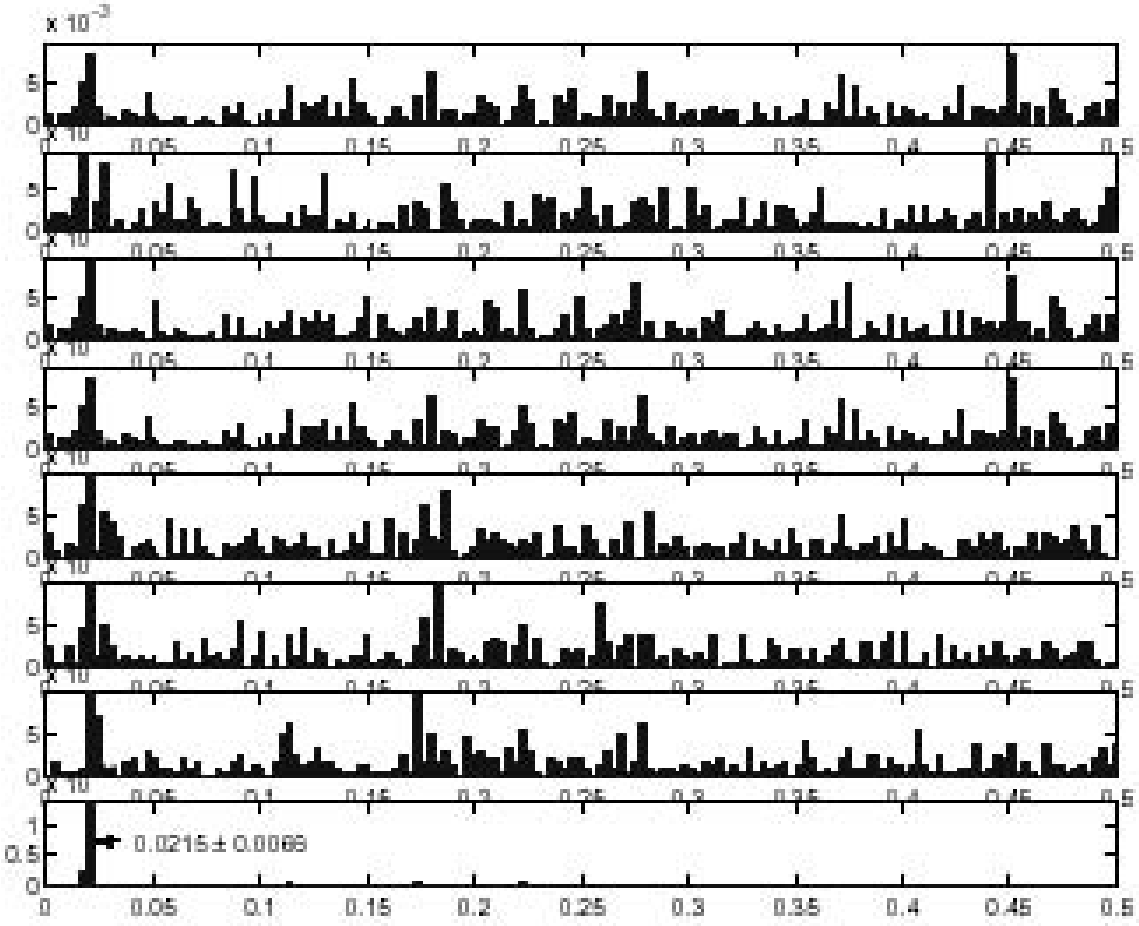


Figure 18. The RRM power spectra and the cross power spectrum of the seven proteins listed in Table 2. The first seven plots are the power spectra for each of the seven Y-box binding proteins. The last plot is the cross power spectra. The prominent peak around 0.0215 ± 0.0066 is the characteristic frequency. The digital resolution was computed as 1/151=0:0066 since the length of the shortest protein is 151.

Similarly, the RRM power spectra and the cross power spectrum of the three promoter sequences of MDR1 genes are shown in Figure 19. A prominent peak appears at 0.0258 ± 0.0033 in the cross power spectrum (the last plot). For the sequences tested, the YB-1 proteins and MDR1 promoters share a common characteristic frequency around 0.0215, suggesting a possible direct interaction between the protein and the promoter sequence.

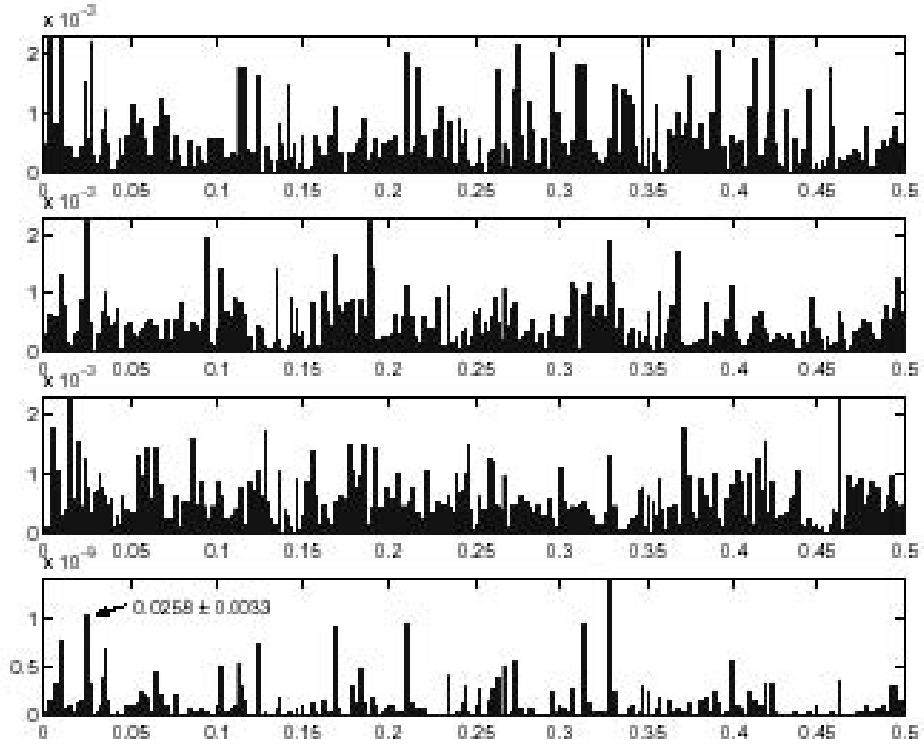


Figure 19. The RRM power spectra and the cross power spectrum of the three promoter sequences of MDR1 genes. The first three plots are the respective power spectra for the three promoters. The last plot is their cross power spectrum. One prominent peak appears at 0.0258 ± 0.0033 in the last plot. The digital resolution is 0.0033.

On the other hand, there was no association between the level of YB-1 expression and Pgp expression status. The median Ckyb-1 immunoreactive score for Pgp negative tumours is 4.0 (interquartile range 2.0 to 5.5) and that for Pgp positive tumours is 5.0 (2.0 to 5.75). There was no statistically significant difference between the two groups when compared with the Mann-Whitney test ($p = 0.446$). The median Frgy-1 immunoreactive score for Pgp negative tumours is 4.0 (interquartile range 1.0 to 5.0) and that for Pgp positive tumours is 3.5 (1.25 to 5.0). Again, there was no difference between the two

groups when compared with the Mann-Whitney test (p = 0.647). The proportion of Pgp positive tumours appear to be greater in higher levels of YB-1 expression (Table 12), but the trend was not large enough for significance.

Table 12. Pgp expression in breast cancers with different levels of YB-1 expression.

	YB-1 expression level		
	Low	Moderate	High
Frgy-1 immunoreactive score (p = 0.555)			
Pgp negative	10	13	10
Pgp positive	6 (38%)	9 (41%)	9 (47%)
Ckyb-1 immunoreactive score (p = 0.807)			
Pgp negative	11	14	8
Pgp positive	7 (39%)	11 (44%)	6 (43%)

Of the 11 tumours with nuclear localization of YB-1, 5 were in this group of tumours for which Pgp immunohistochemistry was performed. All 5 were Pgp positive (p = 0.011); 37% of tumours (19 of 52) without nuclear localization of YB-1 were Pgp positive as well.

3.8 Association between GST-pi and YB-1 expression

It was noticed that the proportion of GST-pi positive tumours was larger with increased YB-1 expression level (Table 13). Using Frgy-1 antibody, 43% of tumours with low YB-1 expression were GST-pi positive compared to 56% and 68% of those with moderate and high YB-1 expression, respectively. There was a statistically significant trend with p

value of 0.048. A similar trend could be seen using the Ckyb-1 immunoreactive scores. However, the trend was of borderline statistical significance (p = 0.060).

Table 13. GST-pi expression in breast cancers with different levels of YB-1 expression.

	YB-1 expression level		
	Low	Moderate	High
Frgy-1 immunoreactive score (p = 0.048)			
GST-pi negative	16	15	12
GST-pi positive	12 (43%)	19 (56%)	25 (68%)
Ckyb-1 immunoreactive score (p = 0.060)			
GST-pi negative	15	18	10
GST-pi positive	12 (44%)	22 (55%)	22 (69%)

3.9 Association between GST-pi and Pgp expression

It was also found that Pgp expression was associated with GST-pi positivity in the same tumour (Table 14). Seventeen (30%) tumours are both GST-pi positive and Pgp positive and 19 (33%) tumours are both GST-pi negative and Pgp negative (p = 0.033).

Table 14. Association between Pgp positivity and expression of GST-pi but not Bcl-2

	Number Pgp positive	Number Pgp negative	p value
GST-pi expression			
Positive	17	14	
Negative	7	19	0.033
Bcl-2 expression			
Positive	11	10	
Negative	13	23	0.274

When analyzed by multivariate analysis using logistic regression, only Pgp expression was found to be associated with GST-pi positivity ($p = 0.041$), whereas there was no association with Frgy-1 or Ckyb-1 immunoreactive score.

3.10 Evaluation of Bcl-2 expression with YB-1 and Pgp expression

Possible association between YB-1 and Bcl-2 expression was also explored, but there was no significant trend in the proportion of Bcl-2 tumours with increasing levels of YB-1 expression ($p = 0.284$ and 0.166 for Ckyb-1 and Frgy-1 immunorective scores, respectively). Bcl-2 expression was not associated with Pgp positivity ($p = 0.274$).

3.11 Metallothionein (MT) expression

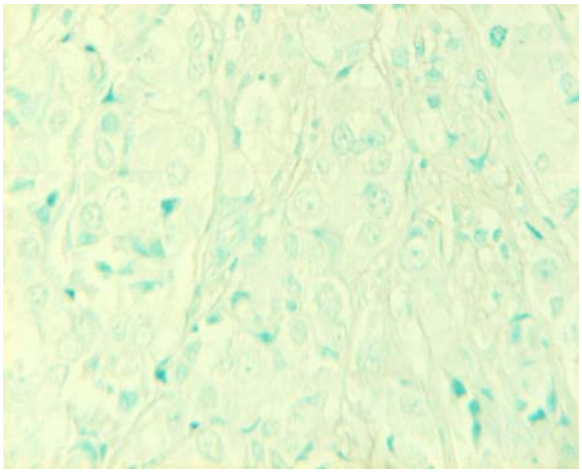
MT was positive in 87 tumours. Amongst the MT positive tumours, the percentage of positive cells had a mean of 30.0% with standard deviation of 25.7%. The MT immunoreactive score ranged from 0 to 285 with a median of 49.8 (Table 15), indicating a positive skew in the distribution of immunoreactive scores.

There were two different patterns of MT expression: predominantly cytoplasmic and predominantly nuclear. 21% (21) of the tumours had the latter pattern of MT localization. Figure 20 illustrates the contrast between these two staining patterns.

Table 15. Statistical distribution of MT immunoreactive score

	MT immunoreactive score
Minimum	0
25^{th} percentile	10
Median	49.8
75^{th} percentile	90
Maximum	285

Higher levels of MT protein expression was associated with a poorer histological grade ($p = 0.009$). The level of MT expression was not different between the groups of breast cancers with different tumour size, estrogen receptor status, regional lymph node status and patient age (Table 16). There were also no significant associations between the protein localization pattern and any of the clinicopathological factors. Further, MT protein expression levels was not significantly different between GST-pi positive and negative ($p = 0.88$), Bcl-2 positive and negative ($p = 0.92$), and Pgp positive and negative breast cancers ($p = 0.83$). There was also no correlation between MT immunoreactive score and Frgy-1 or Ckyb-1 immunoreactive scores ($p = 0.67$ and $p = 0.55$, respectively). Figure 21 shows scatter plots revealing the distribution of MT scores with Frgy-1 and Ckyb-1 scores



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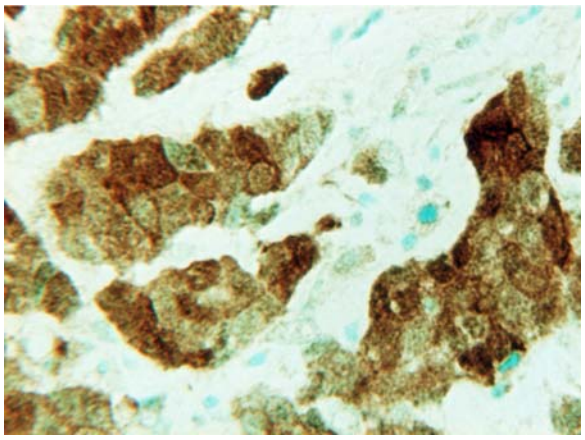

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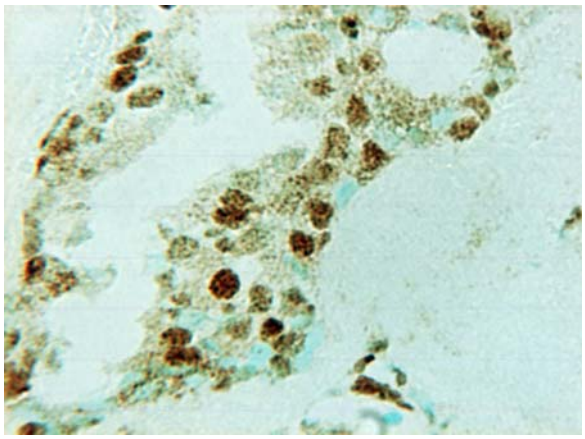
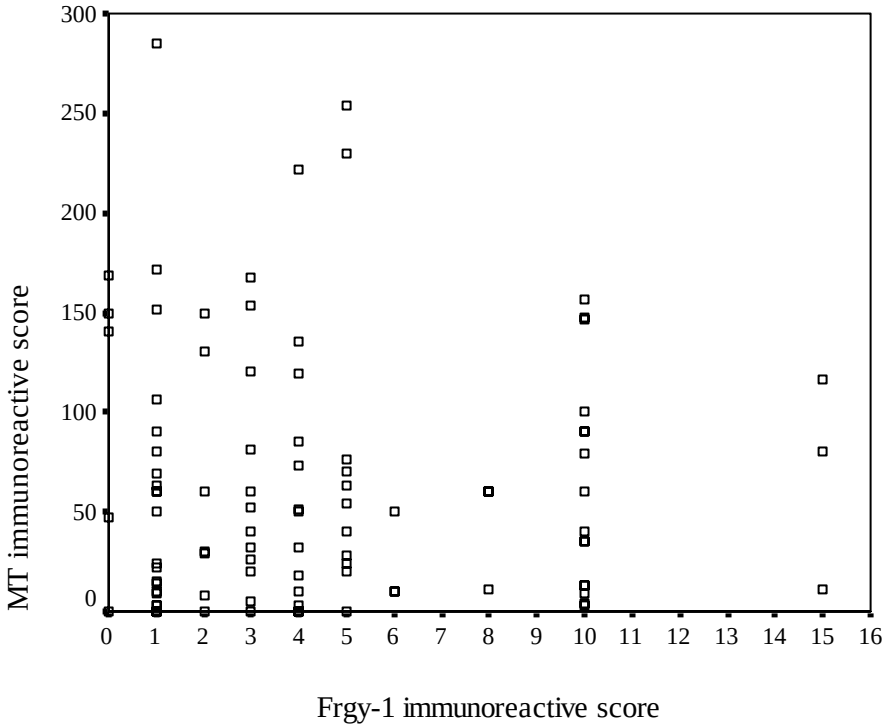


(C)

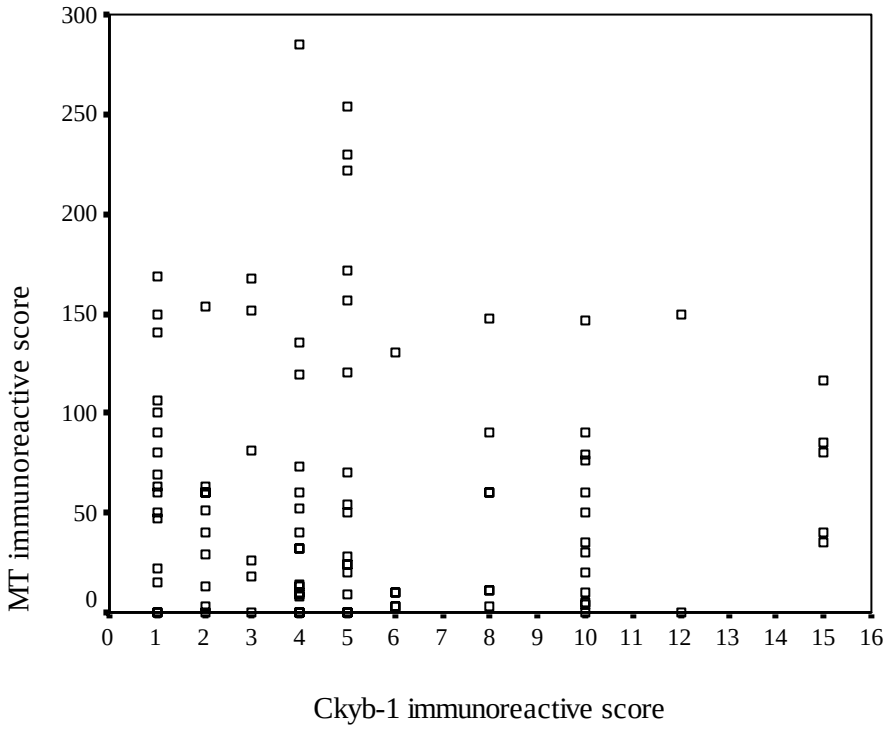
Figure 20. Two different patterns of MT expression detected immunohistochemically: **(A)** negative control, **(B)** predominantly cytoplasmic, and **(C)** predominantly nuclear localization (magnification 160x)

Table 16. MT protein expression levels in different subgroups of breast cancers.

Factors	MT immunoreactive score (median)	Number with cytoplasmic localization	Number with nuclear localization
Patient's age			
50 years and below	35	34	9
More than 50 years	56	44	12
p value	0.36		1.00
Primary tumour			
T1 and T2	44	68	20
T3 and T4	70	10	1
p value	0.27		0.449
Regional lymph node metastasis			
Negative	51	35	12
Positive	38	41	9
p value	0.27		0.461
Estrogen receptor			
Positive	50	28	5
Negative	60	50	14
p value	0.36		0.591
Histological grade			
I and II	28	34	13
III	57	44	8
p value	0.009		0.149
GST-pi expression			
Negative	50	31	12
Positive	45	47	9
p value	0.88		0.215
Bcl-2 expression			
Negative	45	48	12
Positive	50	30	9
p value	0.92		0.803
Pgp expression			
Negative	60	23	10
Positive	38	20	4
p valute	0.83		0.149



(A)

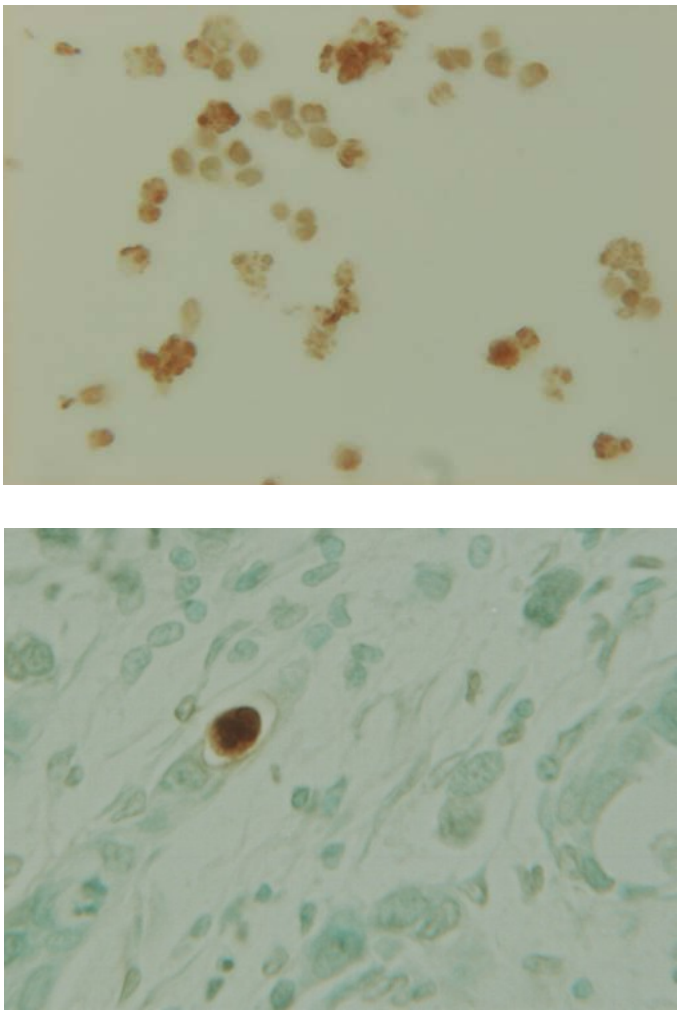


(B)

Figure 21. No linear correlation between MT immunoreactive scores and **(A)** Frgy-1 immunoreactive score (p = 0.67) and **(B)** Ckyb-1 immunoreactive score (p = 0.55)

3.12 Apoptosis

Apoptotic nuclei were detected using the TUNEL method (Figure 22) and the frequency of apoptosis is quantified by the apoptotic index. Apoptotic index ranged from 0 to 9 with a mean of 1.32 ± 0.15. The statistical distribution is shown in Table 17.



(A)

(B)

Figure 22. Apoptotic cells detected by TUNEL **(A)** Positive control of apoptotic HL60 promyelocytic leukemia cells, alongside **(B)** apoptosis detected in breast cancer tissue. (magnification 250x)

Table 17. Statistical distribution of apoptotic index

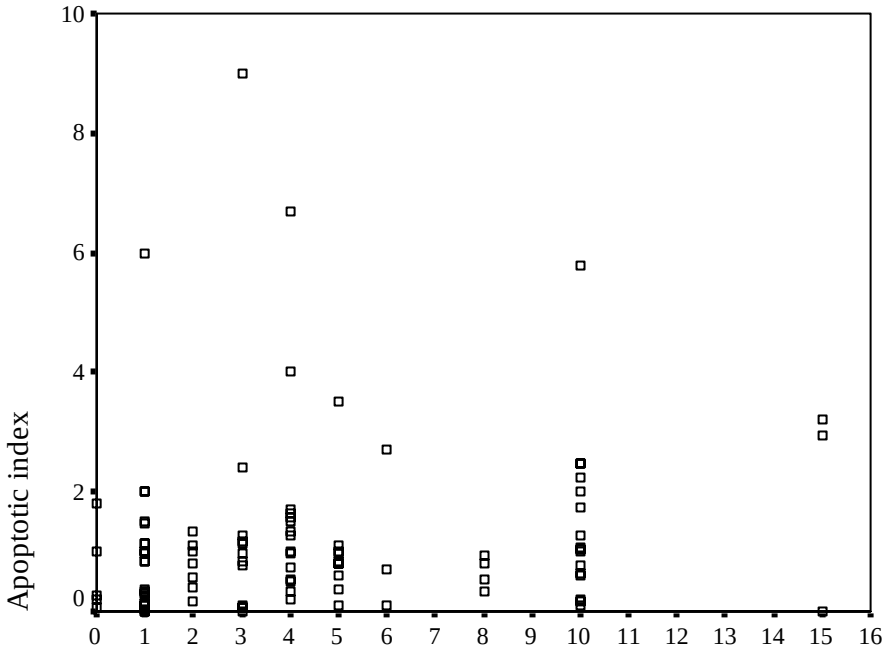
	Apoptotic index
Minimum	0
25^{th} percentile	0.34
Median	0.94
Mean	1.32
Standard error	0.15
Standard deviation	1.59
75^{th} percentile	1.51
Maximum	9.00

Table 18. Mean apoptotic index in relation to clinicopathological factors

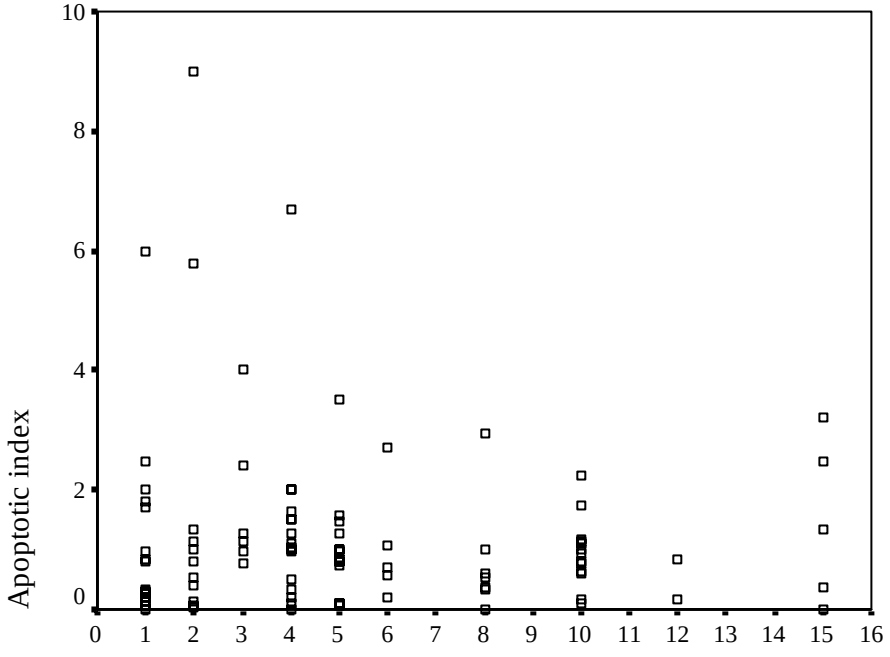
Factors	Apoptotic index (mean ± SEM)
Patient's age	
50 years and below	1.22 ± 0.22
More than 50 years	1.40 ± 0.20
p value	0.547
Primary tumour	
T1	0.94 ± 0.29
T2, T3 and T4	1.40 ± 0.17
p value	0.236
Regional lymph node metastasis	
Negative	1.21 ± 0.20
Positive	1.42 ± 0.22
p value	0.439
Estrogen receptor	
Positive	1.27 ± 0.21
Negative	1.49 ± 0.20
p value	0.486
Histological grade	
I and II	0.97 ± 0.17
III	1.62 ± 0.23
p value	0.029

Histological grade I and II tumours had lower apoptotic indices as compared to the case of Grade III tumours ($p = 0.029$). There was no correlation of the apoptotic index with tumour size, axillary lymph node positivity or estrogen receptor status (Table 18).

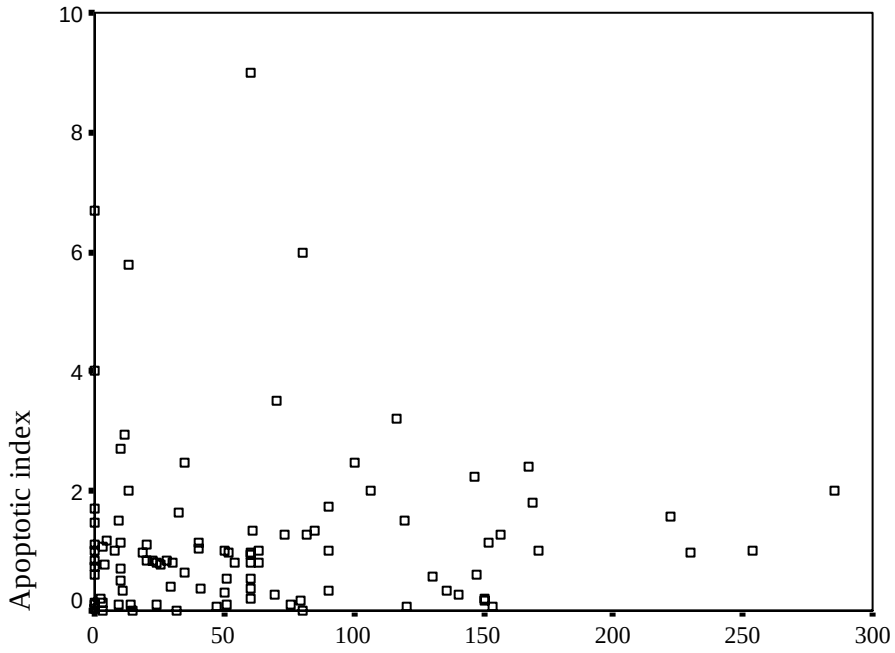
Apoptotic index was not correlated with the immunoreactive scores of MT ($p = 0.311$), Ckyb-1 ($p = 0.897$) and Frgy-l ($p = 0.059$). Interestingly, GST-pi–positive tumours and Bcl-2–positive tumours had significantly lower apoptotic indices compared with the case of their negative counterparts (Table 19). However, when analyzed by multivariate analysis, only histological grade and Bcl-2 immunoreactivity were found to be correlated with apoptosis ($p = 0.008$ and $p = 0.015$, respectively), whereas there was no association between GST-pi immunoreactivity and apoptosis ($p = 0.18$).


Apoptotic index
Frgy-1 immunoreactive score

(A)


Apoptotic index
Ckyb-1 immunoreactive score

(B)



(C)

Figure 23. Scatterplots showing distribution of apoptotic index in tumours of different **(A)** Frgy-1, **(B)** Ckyb-1 and **(C)** MT immunoreactive scores. No linear correlation was found.

Table 19. Mean apoptotic index in relation to protein expressions

Factors	Apoptotic index (mean ± SEM)
GST-pi	
Positive	1.05 ± 0.14
Negative	1.72 ± 0.30
p value	0.024
Bcl-2	
Positive	0.82 ± 0.12
Negative	1.60 ± 0.22
p value	0.011
Pgp	
Positive	0.76 ± 0.12
Negative	1.25 ± 0.23
p value	0.092
MT	
Nuclear	1.21 ± 0.44
Cytoplasmic	1.20 ± 0.14
p value	0.951

3.13 Lipid peroxidation

TBARS assay was used to quantify the level of lipid peroxidation, the effect of oxidative stress on cellular lipids. In the breast cancer tissues, median TBARS level was 116.5 nmol/g wet weight with a range of 18 – 298 nmol/g wet weight. The median TBARS level for breast cancers from older patients more than 50 years of age were significantly higher compared to that of the younger patients ($p = 0.006$). There were no differences in the level of TBARS between the subgroups of breast cancer stratified according to the other common clinico-pathological characteristics (Table 20).

Table 20. Median TBARS level in relation to protein expressions

Clinicopathological characteristics	*N*	TBARS level (median)	p value
Age			
50 years or less	5	78	
51 years or more	27	164	0.006
Lymph node metastasis			
Absent	12	85	
Present	20	124	0.654
Estrogen receptor			
Absent	11	131	
Present	21	98	0.184
Grade			
I and II	17	97	
III	15	154	0.113
Size of tumour			
2cm or less	7	130	
More than 2cm	25	116	0.802

TBARS levels in breast cancers were actually positively correlated with patient age (*rho* = 0.407, p = 0.021). The scatter-plot of patient age against TBARS level is shown in Figure 24.

The median level of TBARS was also higher in GST-pi positive tumours (180 nmol/g wet weight; inter-quartile range 103 to 217) as compared to GST-pi negative tumours (58 nmol/g wet weight, inter-quartile range 39 to 130). The difference is statistically significant (p = 0.009).

For GST-pi positive tumours, higher GST activity was linearly correlated with lower TBARS level (*rho* = -0.535, p = 0.012; Figure 25A). There was no significant correlation between GST activity and TBARS level in GST-pi negative tumours (p = 0.06).

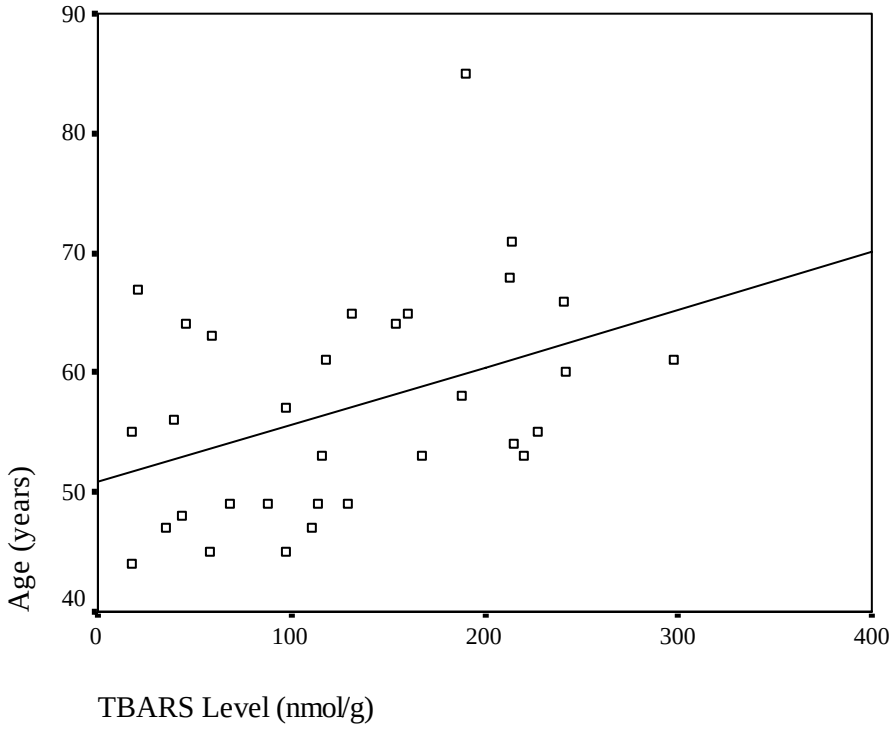
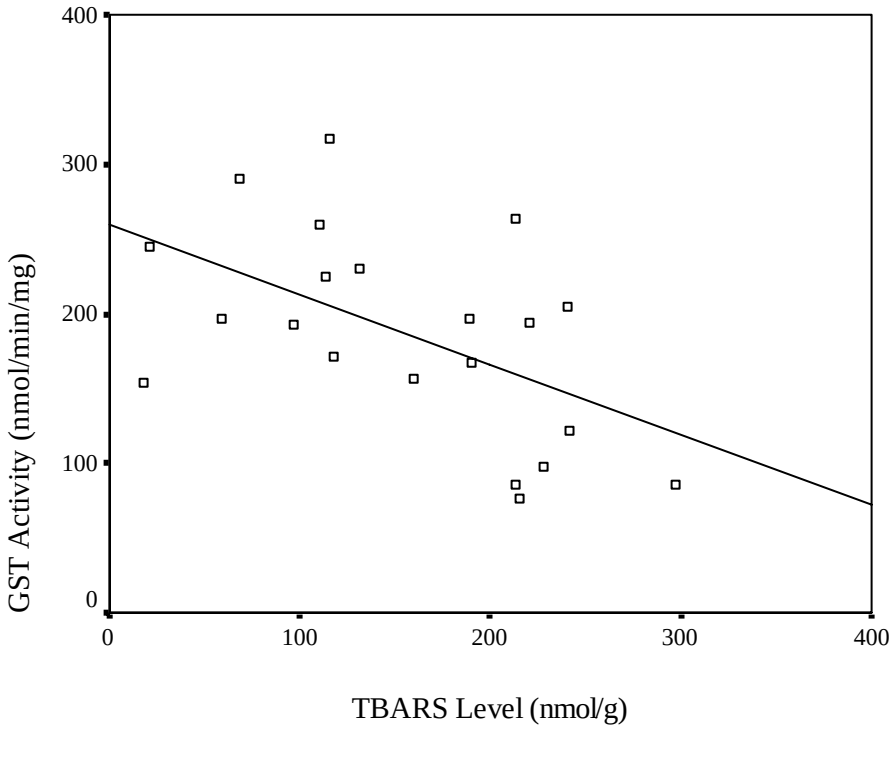
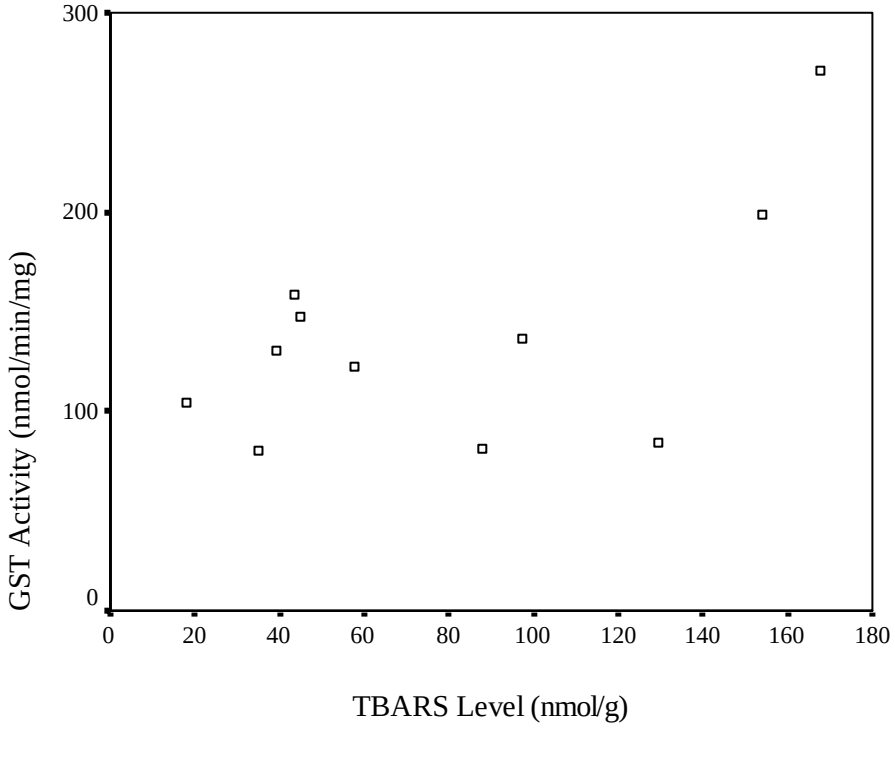


Figure 24. Increased level of oxidative stress (TBARS level) experienced by breast cancer occurring at older ages (*rho* = 0.407, p = 0.021)



(A)



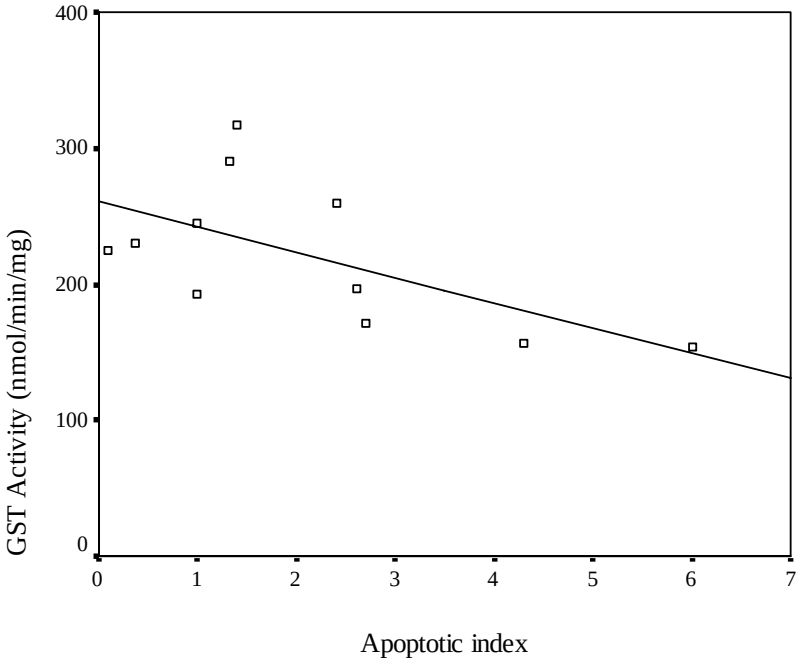
(B)

Figure 25. (A) TBARS level decreases with increasing GST activity in GST-pi positive breast cancers (*rho* = -0.535, p = 0.012), but not in GST-pi negative breast cancers **(B)**.

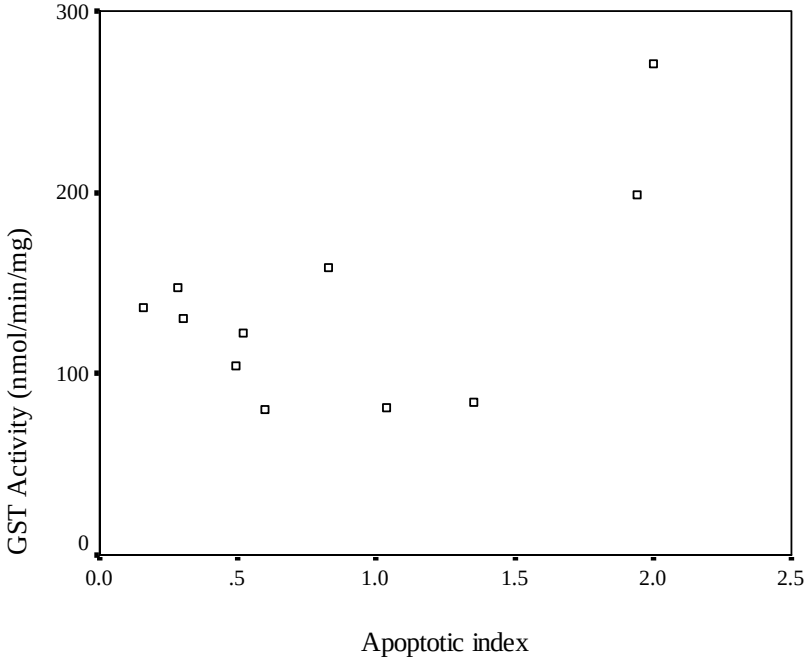
3.14 Effect of lipid peroxidation on association between GST activity and apoptosis

Overall, GST activity in breast cancer was not correlated with apoptotic index ($p = 0.419$). The analysis was followed up by dividing breast cancers into two groups based on the TBARS level. The cut-off level of TBARS was taken to be 175 nmol/g wet weight.

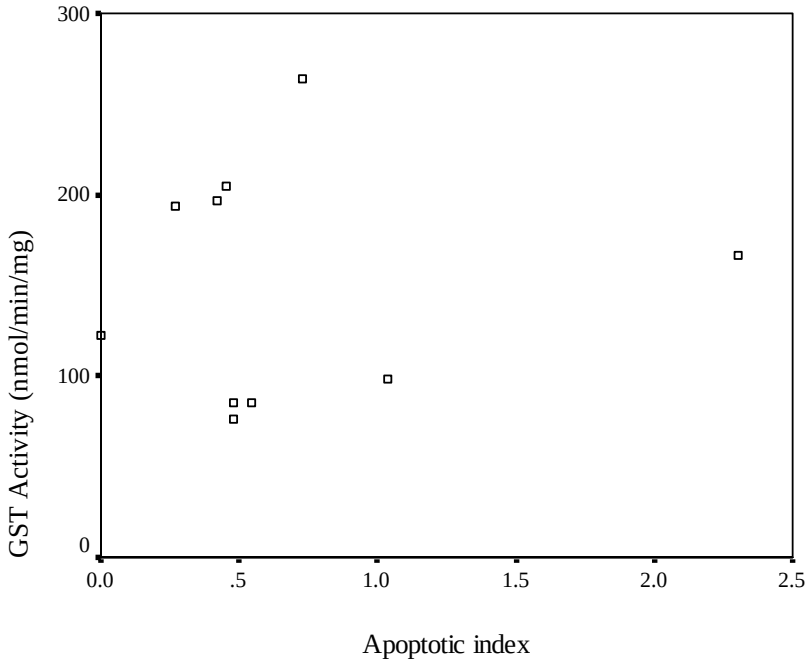
In the group with lower TBARS level, reflecting a lower degree of oxidative stress experienced by the tumours, higher GST activity in breast cancer was associated with a decrease in apoptosis in the subgroup of 11 GST-pi positive tumours (*rho* = -0.607, $p = 0.048$). As illustrated in Figure 26, apoptotic rates in GST-pi positive tumours with higher levels of oxidative stress and GST-pi negative tumours varied independently of GST activity ($p = 0.840$ and 0.066, respectively).


400
300
200
100
0
GST Activity (nmol/min/mg)
0
1
2
3
4
5
6
7
Apoptotic index

(A)


300
200
100
0
GST Activity (nmol/min/mg)
0.0
.5
1.0
1.5
2.0
2.5
Apoptotic index

(B)



(C)

Figure 26. **(A)** Higher GST activity correlates with lower apoptotic index in subgroup of GST-pi positive tumours with lower oxidative stress, TBARS < 175 nmol/g wet weight (*rho* = -0.607, p = 0.048). **(B)** No correlation between apoptotic index and GST activity in GST-pi negative tumours as well as in GST-pi positive tumours with higher oxidative stress experience **(C)**.

3.15 Recurrence-free survival

The patients were followed up for periods ranging from 33 to 1464 days, with a median follow-up of 1117 days. This allowed us to identify aggressive tumours that resulted in early recurrences. Twenty-one patients suffered recurrences, and mean disease-free interval was 981 days.

Just analyzing the established clinico-pathological factors for breast cancer, the number of lymph node metastasis (p = 0.002) and tumour size (p = 0.012) were

significant independent determinants of recurrence, taking into consideration the effects of estrogen receptor status, tumour grade and patient age (Table 21). With all the factors included in the model held equal, every increase in size of tumour by 1cm raises the relative risk of recurrence by 30% (95% confidence interval 5% to 60%) and recurrence risk increases by 9% (95% confidence interval 3% to 16%) for each additional axillary lymph node found to have metastatic tumour.

Table 21. Cox regression analysis of disease free survival of breast cancer patients with common clinico-pathological factors entered into model.

Factors	p value	Hazard ratio
Lymph node metastasis	0.002	1.09
Tumour size	0.012	1.30
Patient age	0.404	-
Grade	0.563	-
Estrogen receptor status	0.402	-

3.15.1 GST-pi

The time to recurrence was significantly affected by GST-pi immunoreactivity ($p = 0.007$), with GST-pi immunopositive tumours having a shorter recurrence-free interval. The Kaplan-Meier survival curves (Figure 27) revealed a significant difference between GST-pi positive and GST-pi negative cases ($p = 0.002$).

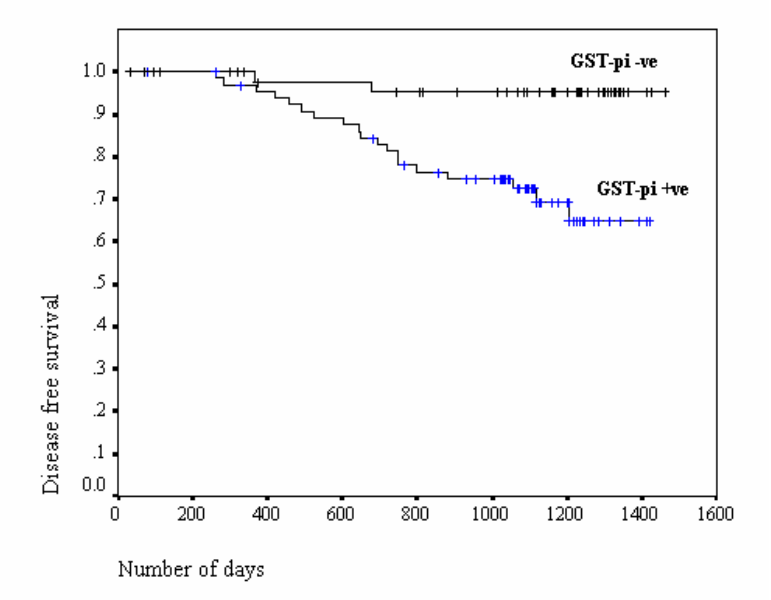


Figure 27. Disease-free survival in patients with GST-pi positive tumours was worse than that of GST-pi negative tumours

Even though the mean number of positive axillary lymph nodes in GST-pi–positive and GST-pi–negative cases was not significantly different (4.0 ± 0.7 versus 6.0 ± 1.1 respectively; p = 0.134), GST-pi immunoreactivity was observed to influence disease-free survival in lymph node–positive cases. (p = 0.004; Figure 28A). Details of this subset of patients with respect to other clinicopathologic parameters are shown in Table 22, suggesting that the prognostic variables were evenly divided amongst the two groups.

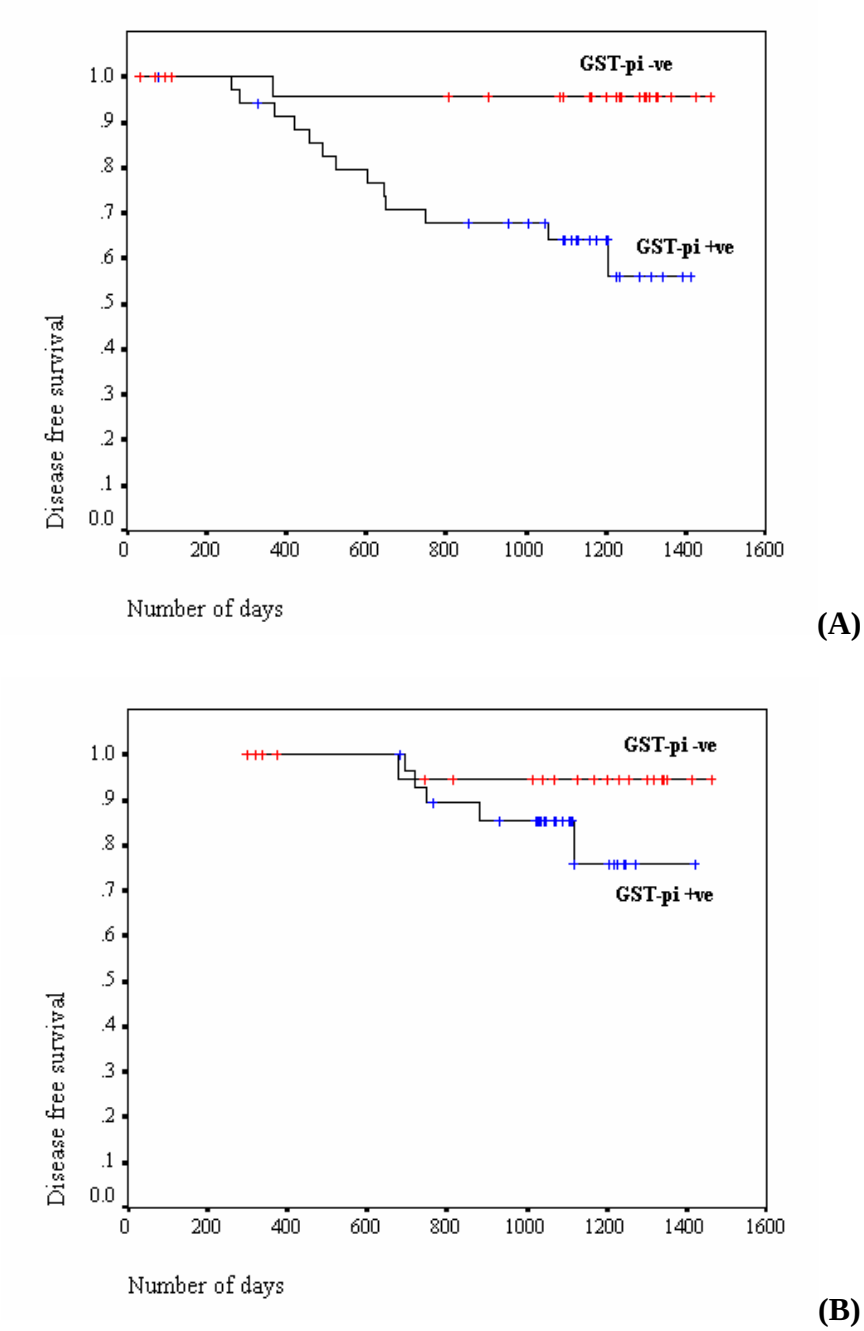


Figure 28. Disease-free survival in node-positive patients **(A)** was significantly associated with GST-pi immunoreactivity (p = 0.004), but **(B)** the difference is not statistically significant in node-negative patients (p = 0.214).

Table 22. Relation of GST-pi expression with clinico-pathological factors in axillary lymph node positive cases.

Clinicopathological characteristics	Number of patients	GST-pi positive	GST-pi negative	p value
Estrogen receptor				0.434
Present	39	18	21	
Absent	23	8	15	
Histologic grade				0.285
I	8	2	6	
II	24	13	11	
III	31	12	19	
Primary tumour				0.652
T1	9	5	4	
T2	42	17	25	
T3 and T4	12	5	7	

3.15.2 Bcl-2

There was no association between Bcl-2 immunoreactivity and recurrence-free survival (p = 0.08). Figure 29 shows the Kaplan-Meier survival curves.

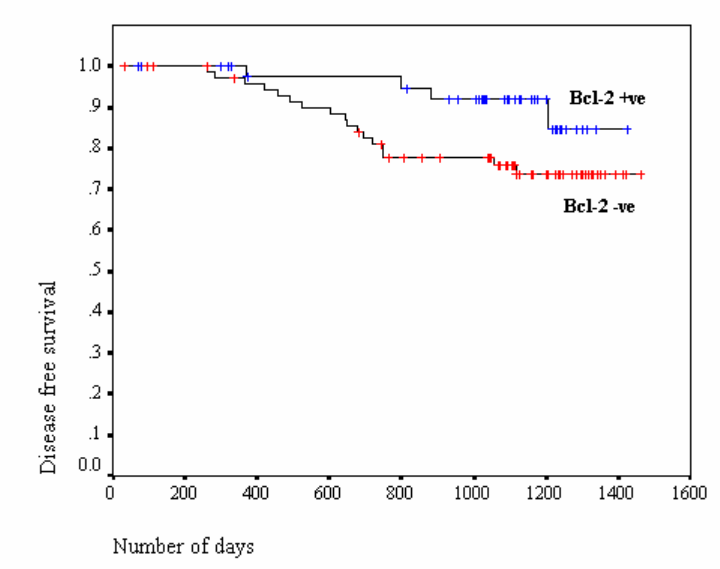


Figure 29. Disease free survival not affected by Bcl-2 status.

3.15.3 YB-1

As can be seen in Figure 30, tumours with higher Ckyb-1 immunoreactive score tended to have a higher recurrence risk compared to those with low expression (nevertheless, p = 0.39). A similar trend could be discerned (Figure 31) if the tumours were grouped according to Frgy-1 immunoreactive score (but p = 0.28). When the tumours with low scores with the other tumours with higher scores are compared, the difference was not statistically significant when the tumours were grouped according to Ckyb-1 score (p = 0.17) and Frgy-1 score (p = 0.11).

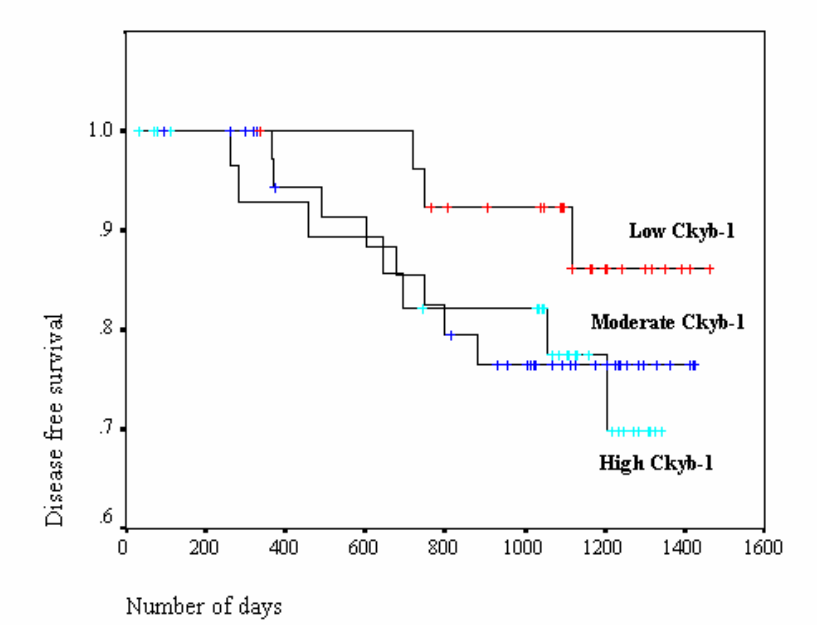


Figure 30. Kaplan-Meier survival curves showing differences in disease free survival between tumours grouped according to Ckyb-1 immunoreactive score.

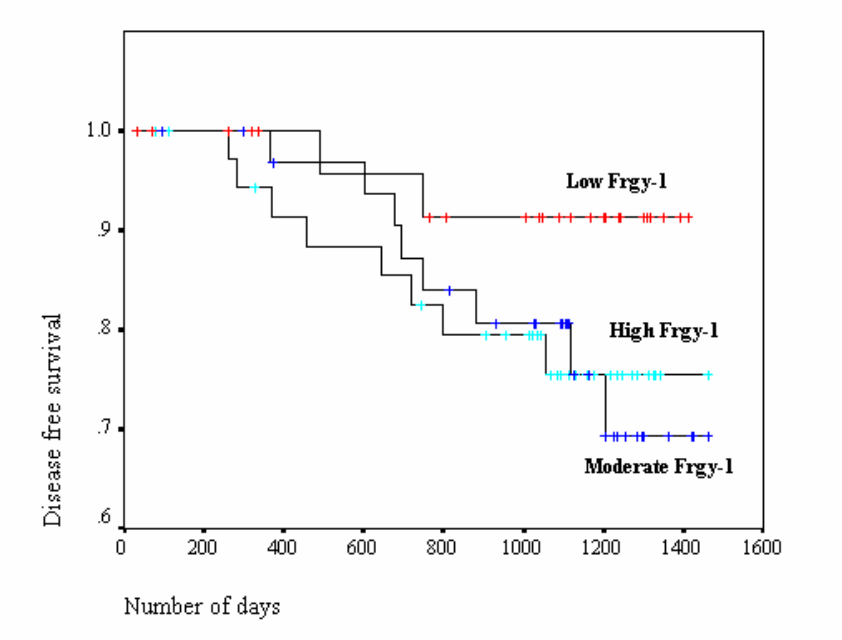


Figure 31. Kaplan-Meier survival curves showing differences in disease free survival between tumours grouped according to Frgy-1 immunoreactive score.

3.15.4 MT

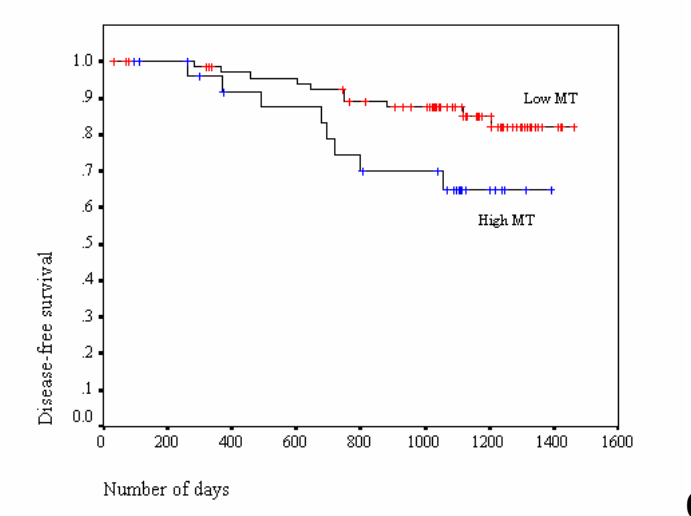
The study of how MT expression affects disease free survival was performed by dividing the breast cancers into two groups based on MT immunoreactive score. The cut-off was selected by entering different cut-offs into a Cox regression model and choosing the most discriminatory cut-off using backward elimination. With this method, MT score of 80 was picked as the cut-off.

Higher MT expression levels in the primary breast tumour resulted in increased recurrence risk ($p = 0.037$). The Kaplan-Meier disease-free survival curve illustrates the difference (Figure 32A).

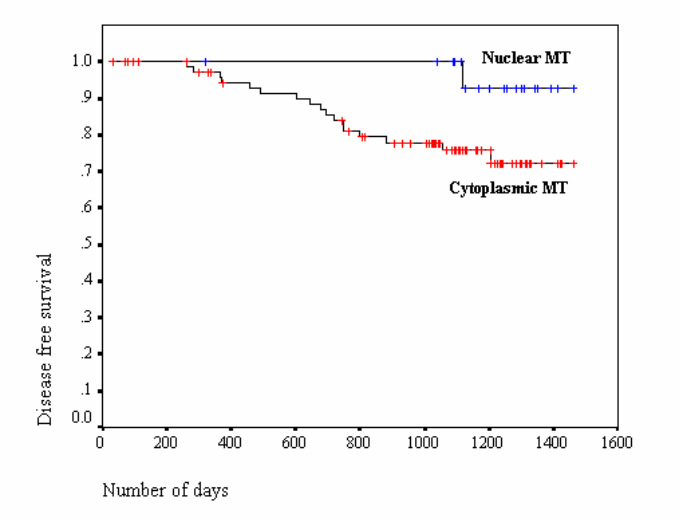
Tumours with predominantly nuclear expression were associated with reduced risk of recurrence ($p = 0.046$) by univariate analysis. Taking into consideration MT expression levels in Cox's proportional hazards model, this association was only of marginal statistical significance ($p = 0.078$). MT immunoreactive scores between the two groups of tumours were not significantly different ($p = 0.23$) by Mann-Whitney test. The statistical distribution of MT score of those with predominantly nuclear localization compared to the tumours with cytoplasmic expression is shown in Table 23.

Table 23. Comparing the statistical distribution of MT immunoreactive score between those with predominantly cytoplasmic expression of MT and those with predominantly nuclear localization.

	MT immunoreactive score	
	Cytoplasmic MT	Nuclear MT
Minimum	0	3
25^{th} percentile	9.8	32
Median	37.5	60
75^{th} percentile	86.3	90
Maximum	285	169



(A)



(B)

Figure 32. Disease free survival of breast cancers increased by **(A)** reduced level of MT expression (p = 0.037) and **(B)** predominantly nuclear expression of MT (p = 0.046)

3.15.5 Multivariate analysis

When clinico-pathological factors influencing recurrence were analyzed with the expression of the protein markers of interest by multivariate analysis, GST-pi status, Bcl-2 status and MT expression level were found to significantly influence disease-free survival

(p = 0.015, 0.038 and 0.030, respectively). Tumour size and the number of metastatic lymph nodes remained independent prognostic factors (Table 24).

The relative risk of tumour recurrence was 9.1 times as high (95% confidence interval 1.56 to 52.7) in patients with GST-pi positive breast tumours as compared to GST-pi negative tumours. Higher MT expression was associated with a relative risk of recurrence of about 3.6 times (95% confidence interval 1.13 to 11.2) that of tumours with lower MT expression.

Conversely, Bcl-2 expression reduced the risk of recurrence in breast cancer patients to about a quarter of that of Bcl-2 negative tumours (95% confidence interval 0.069 to 0.93).

Table 24. Cox's multivariate analysis of disease free survival and relative risk of recurrence in breast cancer patients

Factors	P value	Hazard ratio
Lymph node metastasis	0.012	1.08
Tumour size	0.045	1.32
GST-pi expression	0.014	9.08
Bcl-2 expression	0.038	0.25
Frgy-1 score	0.486	-
Ckyb-1 score	0.883	-
MT localization	0.187	-
MT score	0.030	3.57

3.16 Adjuvant chemotherapy and recurrence

Table 25 shows the treatment regime prescribed for the group of breast cancer patients after surgery stratified according to protein marker expression. Adjuvant chemotherapy

regimes could be broadly divided into two groups: the cyclophosphamide/methotrexate/5-fluorouracil (CMF) regime, and the doxorubicin-based regimes such as doxorubicin/cyclophosphamide, doxorubicin/taxol, and cyclophosphamide/doxorubicin/5-fluorouracil.

Table 25. Treatment regime of breast cancer patients after surgery stratified according to protein marker expression.

Biomarker status	No chemotherapy	With adjuvant chemotherapy	Number on CMF
GST-pi positive	27	40	22
GST-pi negative	18	31	14
Bcl-2 positive	19	24	12
Bcl-2 negative	26	47	24
Pgp positive	0	24	14
Pgp negative	0	33	19
MT high (score > 80)	11	17	7
MT low	31	40	26
Ckyb-1 high (score > 2)	33	39	22
Ckyb-1 low	9	18	11
Frgy-1 high (score >1)	30	41	24
Frgy-1 low	12	16	9

CMF = cyclophosphamide/methtrexate/5-fluorouracil regime

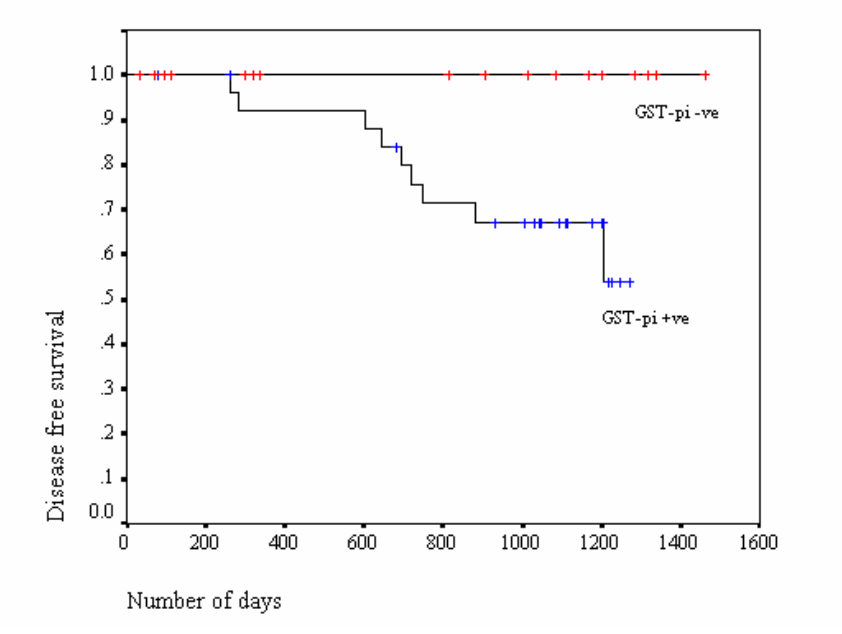
It was then determined if different expression of the various proteins was associated with differences in recurrence-free survival in the group of patients receiving adjuvant chemotherapy. The difference is compared with that of the group of patients who did not receive adjuvant chemotherapy.

3.16.1 GST-pi

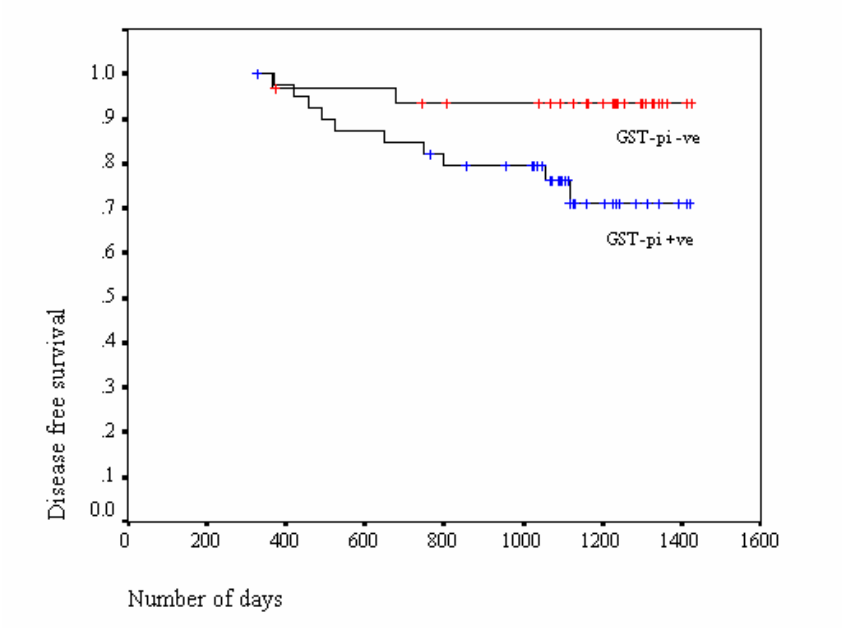
GST-pi expression resulted in poorer disease free survival in both groups of patients who were surgically treated only ($p = 0.02$) versus those who received adjuvant chemotherapy in addition to surgery ($p = 0.04$). The Kaplan-Meier survival curves for GST-pi are illustrated in Figure 33.

3.16.2 Bcl-2

In contrast, the difference in disease free survival was not significantly different in both groups of patients if they were stratified by Bcl-2 expression ($p = 0.14$ and $p = 0.21$ for surgery alone and surgery with adjuvant chemotherapy respectively). The Kaplan-Meier survival curves for Bcl-2 are illustrated in Figure 34.

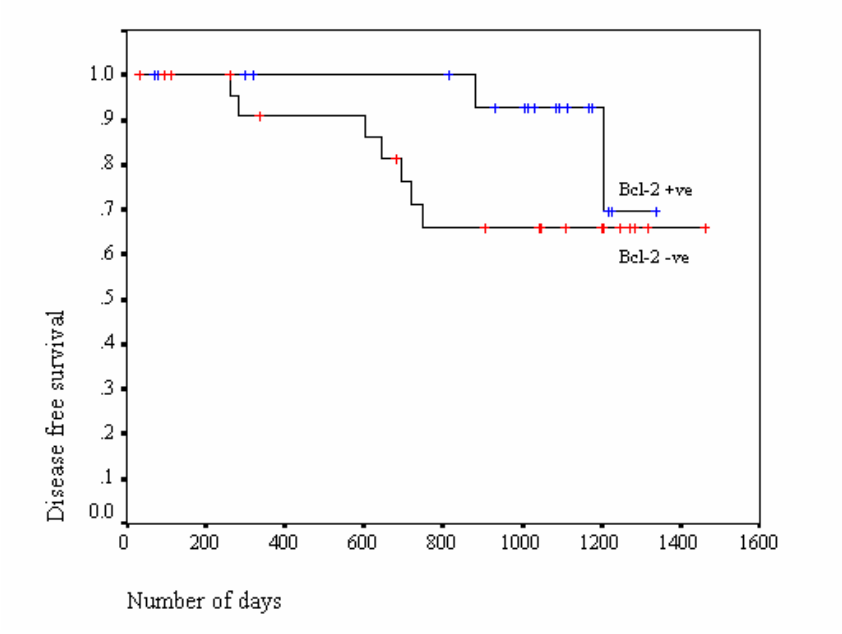


(A)

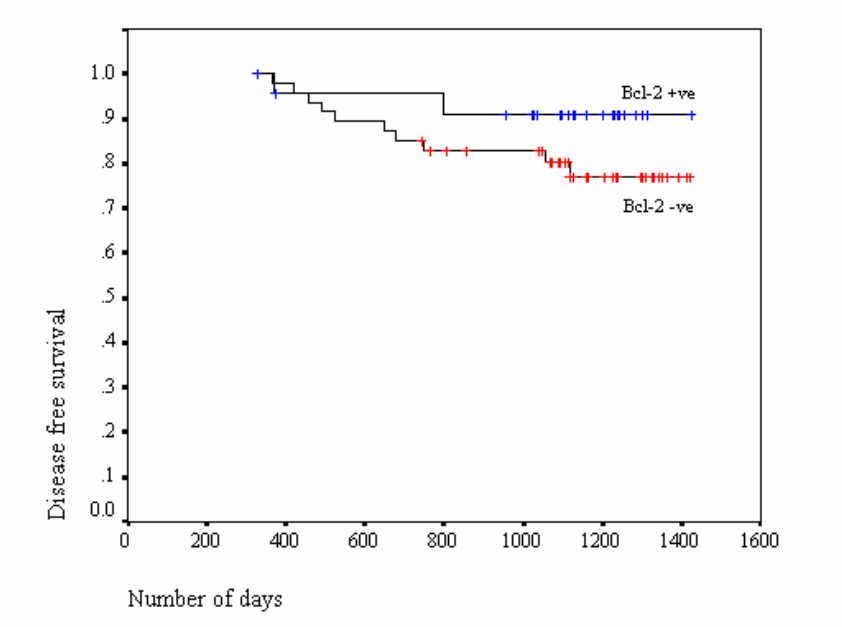


(B)

Figure 33. Disease-free survival with GST-pi positive tumours was inferior to GST-pi negative tumours in both **(A)** patients who received surgery only and **(B)** surgery with adjuvant chemotherapy.



(A)



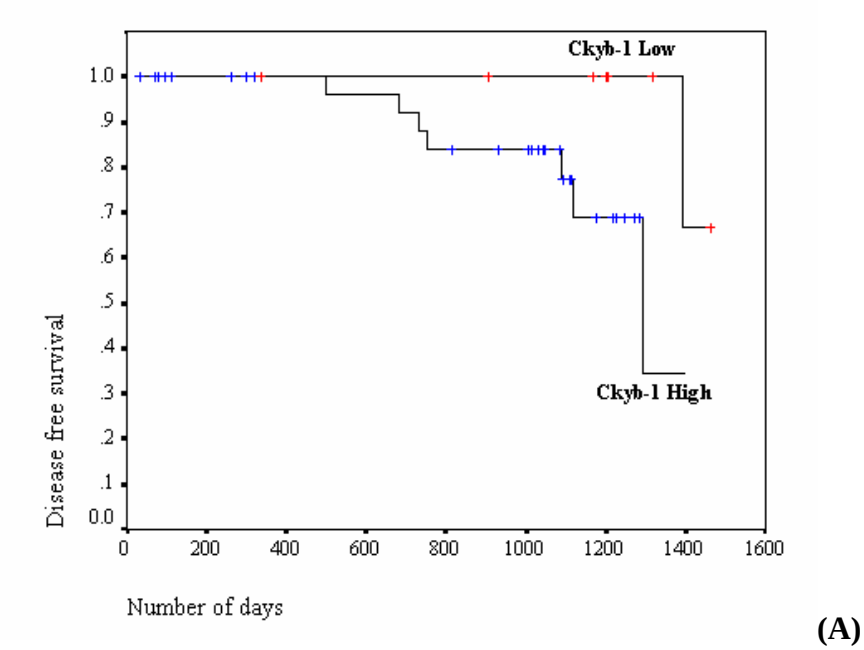
(B)

Figure 34. Disease-free survival with Bcl-2 positive tumours was no different from Bcl-2 negative tumours in both **(A)** patients who received surgery only and **(B)** surgery with adjuvant chemotherapy.

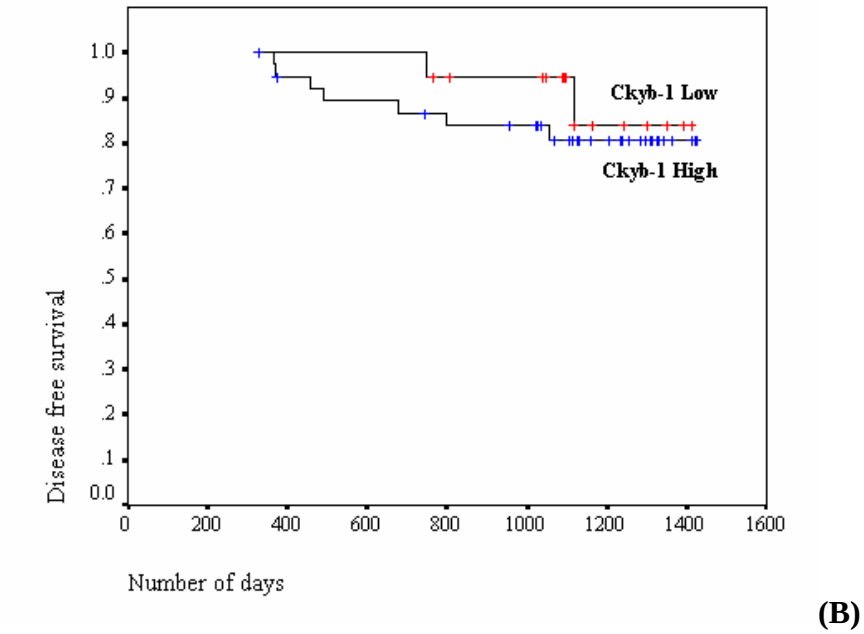
3.16.3 YB-1

In the group of patients who did not receive adjuvant chemotherapy, only 1 of 8 patients (13%) with low Ckyb-1 immunoreactive score in the primary tumour experienced disease recurrence, whilst 5 of 18 patients (28%) with high Ckyb-1 score recurred. The difference in recurrence-free survival between the breast cancers of low YB-1 expression and that of higher (moderate to high) expression was significant ($p = 0.034$; Figure 35A) in this group of patients. YB-1 expression did not affect disease-free survival in the patients who received adjuvant chemotherapy ($p = 0.70$).

A similar association with disease free survival could be seen using Frgy-1 score. In the group of patients who did not receive adjuvant chemotherapy, only one of 12 patients (8.3%) with low Frgy-1 score in the primary tumour experienced disease recurrence, compared to 4 of 18 patients (22%) with high Frgy-1 score. However, the difference in recurrence-free survival between the breast cancers of lower YB-1 expression and that of higher expression was only statistically significant if the Frgy-1 score cut-off was defined as 3, instead of using the 25^{th} percentile cut-off as in previous analyses ($p = 0.048$, Figure 36). YB-1 expression did not affect disease-free survival in the group of patients who received adjuvant chemotherapy ($p = 0.21$, comparing patients with low Frgy-1 score against those patients with moderate or high Frgy-1 score).



(A)



(B)

Figure 35. Disease-free survival of tumours with higher Ckyb-1 scores was poorer than those of low Ckyb-1 scores in **(A)** patients who received surgery only, but **(B)** not in patients who received both surgery and adjuvant chemotherapy.

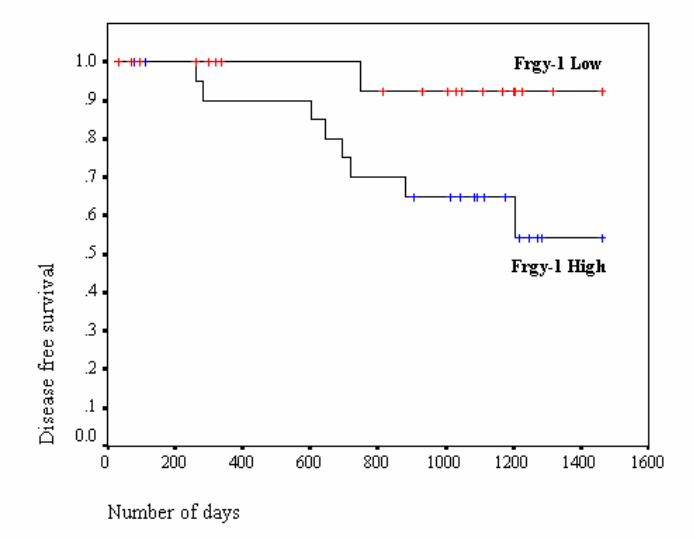


Figure 36. Disease-free survival of tumours with higher Frgy-1 scores was poorer than those of lower Frgy-1 scores in patients who did not receive adjuvant chemotherapy. Statistical significance only if a score cut-off of 3 was used to separate the two groups.

Among the 14 patients with high Ckyb-1 immunoreactive score and administered chemotherapy, 2 of 7 (29%) receiving anthracycline-based chemotherapy recurred compared to no recurrence amongst patients receiving the CMF regime. While there was no difference in Ckyb-1 score between all patients receiving CMF compared to anthracycline based regimes ($p = 0.96$), patients who developed recurrence despite being on the CMF regime, have a lower YB-1 score (range 2 to 5) compared to that of patients with recurrence after receiving anthracycline-based therapy (YB-1 score range 5 to 10; $p = 0.024$). Table 26 shows the details illustrating the point. A similar pattern was seen if Frgy-1 scores were considered, although it did not reach statistical significance. Amongst the 19 patients with high Frgy-1 score, 3 of 9 (33%) receiving anthracycline-based

chemotherapy recurred compared to 1 recurrence out of 10 (10%) patients receiving the CMF regime.

3.16.4 Pgp

Similarly, Pgp over-expression was not significantly associated with disease-free survival ($p = 0.34$) in the group of patients who received adjuvant chemotherapy. Considering just the patients who received anthracycline-based chemotherapy regimes, 1 of 14 (7%) Pgp negative tumours recurred compared to 3 of 10 (30%) tumours in the Pgp positive group. The difference was not statistically significant ($p = 0.27$). Table 26 shows how Pgp expression relates to type of chemotherapy treatment in patients who developed recurrence during follow-up. The Kaplan-Meier survival curves for Pgp expression are illustrated in Figure 37.

Table 26. YB-1 and Pgp status of breast cancer patients with recurrence after chemotherapy.

Patient's Serial no.	Chemotherapy regime	Ckyb-1 immunoreactivity score	Pgp over-expression
23768	C	2	-
30071	C	2	-
5204	C	4	+
31674	C	4	-
23471	C	5	+
7840	A	5	+
21936	A	5	+
16048	A	8	+
19001	A	10	-

C = CMF chemotherapy
A = Anthracycline-based chemotherapy

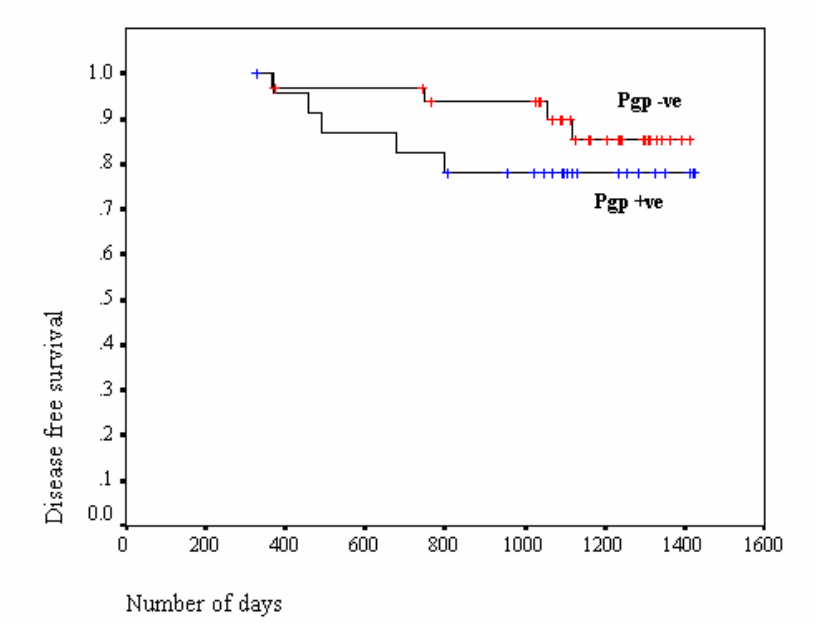
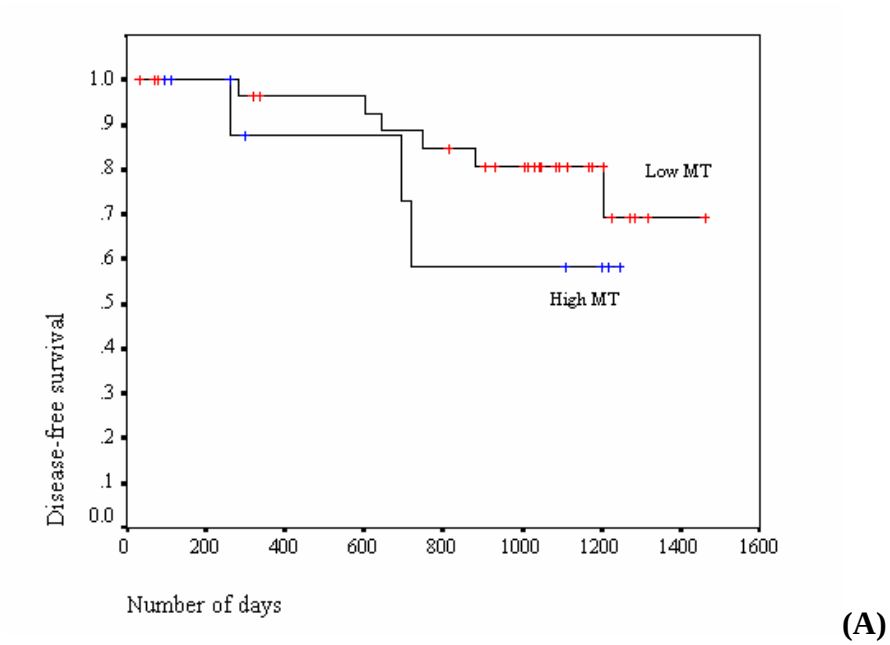


Figure 37. Kaplan-Meier survival curve showing no statistical significance in the difference in disease-free survival of patients having Pgp positive tumours compared to those having Pgp negative tumours (p = 0.31)

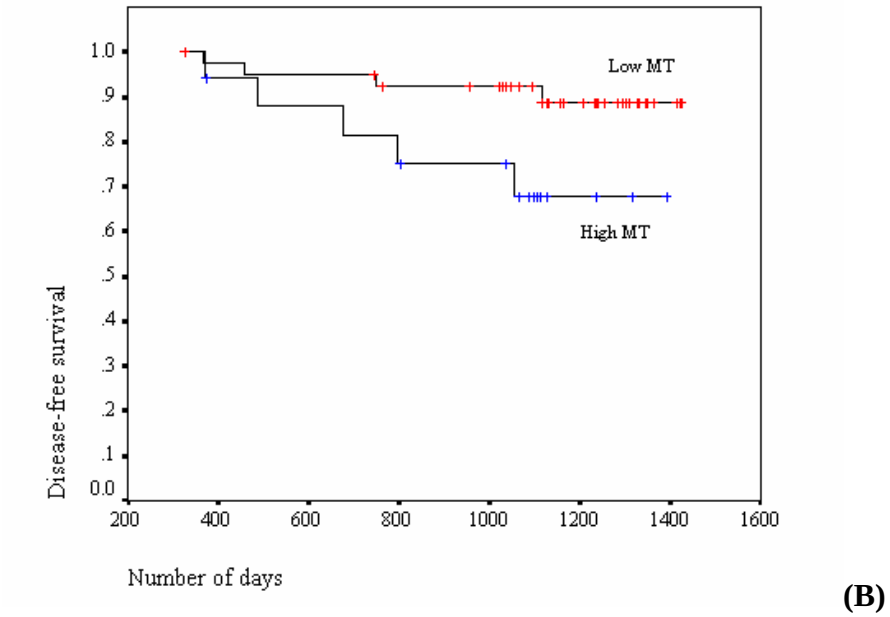
3.16.5 MT

MT expression had a different effect on disease free survival in the two treatment groups compared to Bcl-2 and GST-pi expression.

The difference in recurrence-free survival was more prominent amongst the group of patients who underwent chemotherapy (p = 0.048), whereas in the group of patients not prescribed chemotherapy, recurrence-free survival of patients with low MT expression were not substantially more favourable compared to their high MT expressing counterparts (p = 0.28). Figure 38 illustrates the survival curves.



(A)



(B)

Figure 38. Disease-free survival of tumours with high levels of MT expression was no different from those of low levels of MT expression in **(A)** patients who received surgery only, but **(B)** was associated with poorer prognosis in patients who received both surgery and adjuvant chemotherapy.

Discussion

4.1 GST-pi expression in breast cancer

Tumour aggressiveness is an important issue in breast cancer. Even when the tumour can be removed surgically from the breast and axillary lymph nodes, micrometastasis may remain in the body and give rise to overt disease after a period of time, possibly years (Ozbas *et al.*, 2003). With clinically detectable breast cancers just localized to the breast, about 30 to 35% of patients will suffer from recurrence after 5 years of follow-up. Use of adjuvant chemotherapy in these patients will reduce the rate of recurrence to about 25% (Early Breast Cancer Trialists' Collaborative Group, 2003).

The substantial percentage of recurrence even after adjuvant chemotherapy highlights the critical role in identifying breast cancer patients at high risk of such events. Whilst hundreds of oncogenic changes occur in breast cancers (Liu, 2003), there remains a need to identify suitable tumour markers.

GST-pi expression was detected in 58% of breast cancers in this group of Asian breast cancer patients. The frequency of expression is comparable to Caucasian populations where 40 to 56% of breast cancers have been reported to be GST-pi positive (Murray *et al.*, 1993 and Silvestrini *et al.*, 1997). In the present study, tumours over-expressing GST-pi had higher total GST activity, suggesting that GST-pi contributes significantly to variation of GST activity in breast cancer.

The control of GST-pi expression is through a number of cis-acting regulatory elements located 5' to the gene: an anti-oxidant responsive element (ARE) mediating responsiveness to phenolic antioxidants; a TPA response element (TRE) that is activated

by AP-1 (the c-Fos/c-Jun containing transcription factor complex); and a GC box providing basal promoter activity (Jhaveri *et al*., 1998[a]).

Up-regulation of GST-pi is mediated by a TRE-like enhancer in mouse hepatocarcinogenesis (Sakai, 1990). More recently, 2 cAMP responsive elements (CRE) were found, one distal and the other proximal to the transcriptional start site. It appears that GSTP1 (gene coding for GST-pi) regulation in MCF-7 breast cancer cell lines is mediated by cAMP via the TRE and proximal CRE sites (Lo *et al.*, 2001).

In a significant proportion of human breast (30%) and renal (20%) cancers, GST-pi expression is suppressed by hypermethylation of the GC promoter (Esteller, 2000). Indeed, most of the peri-tumoral benign breast epithelium in this study sample shows GST-pi expression (74%), and 56% of those are associated with GST-pi negative breast cancers. The reason for this epigenetic change to suppress GST-pi expression in breast cancers is still a subject of speculation. It was theorized that the suppression of GST-pi expression actually promoted tumour formation: estrogens may be metabolized into electrophilic intermediates (GST-pi substrates) that covalently bind DNA, resulting in mutations. Reduced GST-pi expression might lead to accumulation of these genotoxic metabolites. However, correlation between the presence of estrogen-related adducts and the GST-pi epigenetic lesion has not been demonstrated.

On the contrary, the results suggest that GST-pi expression is associated with more aggressive breast cancers with significantly poorer prognosis. Although in the study population, GST-pi expression is not associated with larger primary tumours, poorer histologic differentiation or increased lymph node metastasis, patients with GST-pi positive tumours have an 8-fold higher risk of recurrence and shorter disease-free survival

compared to their GST-pi negative counterparts. Earlier on, Gilbert *et al.* (1993) suggested that increased GST-pi expression could be an important predictor of recurrence and death in node negative breast cancer patients. In this study, it was found that in node positive tumours, GST-pi expression was also associated with higher risk of recurrence compared to GST-pi negative tumours.

In addition, GST-pi expression in the primary tumour increases recurrence risk after adjuvant treatment. Silvestrini and co-workers (1997) found that the risk of local recurrence after loco-regional radiotherapy was higher for patients with tumours exhibiting elevated levels of GST-pi protein. Focusing on patients receiving systemic chemotherapy, this study showed a similar increase in the risk of early recurrence in the group of such patients with GST-pi positive primary tumours, compared to those with GST-pi negative tumours..

4.2 GST-pi and apoptosis

One of the functions of GST-pi in the normal cell is to provide protection against reactive oxygen species (ROS).

ROS includes toxic molecules such as hydroxyl radicals, superoxide anions and hydrogen peroxide. These molecules are produced during cellular metabolism, for example: in the mitochondria, from the partial reduction of oxygen by the electron transport chain; in the endoplasmic reticulum, from NADPH-cytochrome P450 reductase; and in the peroxisome, from the production of hydrogen peroxide that leaks into the

cytoplasm, subsequently reacting with iron(II) or copper(I) to form hydroxyl radicals via Fenton reaction. ROS is also formed from the decomposition of oxyhemoglobin, auto-oxidation of catecholamines, ultra-violet irradiation of tryptophan and breakdown of water in cells by infra-red radiation. ROS may also arise from exogenous sources: leukocytes release ROS in inflammatory reactions and can cause DNA damage of cells nearby (Shacter *et al.*, 1988).

At the biochemical level, ROS reacts with nucleic acids, proteins and lipids. 8-oxo-2'-(de)oxyguanosine is a well-known DNA oxidative product. ROS have also been found to induce single and double-stranded DNA breaks, modifications of the sugar moiety, DNA-protein crosslinks, depurination and depyrimidination. Chemical modification of nucleic acids alters hydrogen bonding specificity, resulting in errors in translation, transcription and DNA replication.

Protein function and membrane integrity are also affected by the reaction of proteins and lipids with ROS (Tamarit *et al.*, 1998). Many proteins contain cysteines that can be oxidized to form inter- and intra-molecular disulfide bonds. Changes in redox conditions in the cell will cause variations in the oxidative state of these amino acids, consequently affecting protein structure, protein-protein and protein-DNA interactions. Some transcription factors interact with DNA via a "zinc finger" motif, consisting of four cysteine or histidine surrounding a zinc atom. The cysteine residues are sensitive to redox state variation in the nucleus, resulting in modification of the activity of the transcription factors. For example, the OxyR response element, a DNA transcription regulatory element involved in oxidative stress response, is activated by a transcription factor which can be reversibly inhibited by reduction of a disulfide bond by glutaredoxin I (Zheng *et al.*,

1998). The tumour suppressor protein, p53, has 9 cysteine residues, 4 of which are present in the DNA binding domain. Thiol oxidation is thought to change the structural organization p53, abolishing its interaction with its specific DNA target sequence, but not its non-specific binding to DNA (Parks *et al.*, 1997).

Quantitative analysis of TBARS, breakdown products of lipid peroxidation, was used in the study as a measure of oxidative stress. Amongst breast cancers, there is a substantial variation in oxidative stress experience. The results showed that tissues obtained from older patients had higher levels of TBARS. This may be partly explained by increased exposure to oxidants in the systemic circulation, since assays of markers of oxidative stress in the sera of normal human subjects aged 20 to 70 years showed the same trend (Kasapoglu, 2001). On the other hand, levels of oxidative stress were not influenced by traditional pathological markers of tumour differentiation, such as tumour grade and hormone receptor status.

With its glutathione peroxidase activity and ability to inactivate by conjugation, carbonyl-, peroxide-, and epoxide-containing metabolites produced within the cell by oxidative stress, GST-pi complements the activity of a number of anti-oxidant defences. Biochemical anti-oxidants, such as glutathione, ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E) form the first level of protection. For example, vitamin E scavenges hydroxyl radicals and reacts with peroxy and alkoxy radicals to stop radical chain reactions. Binders, such as transferrin and ferritin for iron, and caeruloplasmin for copper, sequester these ions so that they are not able to catalyze radical forming reactions. Intra-cellular enzymes form the third level of anti-oxidant protection. Such enzymes include superoxide dismutase, glutathione peroxidase, catalase and thioredoxin reductase.

Superoxide dismutase converts superoxide to the less reactive hydrogen peroxide. Catalase breaks down hydrogen peroxide to form oxygen. Glutathione peroxidase catalyzes the reduction of a variety of organic hydroperoxides (such as lipid hydroperoxides) and hydrogen peroxide, using glutathione as the reducing agent.

The cell is dependent on these protective mechanisms to maintain ROS at a tolerable level. Consistent with the fact that GSTP1 expression is regulated by an anti-oxidant responsive element (ARE), it was observed that tumours with up-regulation of GST-pi expression appear to be those that have experienced higher levels of oxidative stress (higher TBARS level).

When the cell experiences excessive oxidative stress, deleterious effects on cell function and survival will result.

ROS has been shown to trigger cell cycle checkpoint responses. Peroxides induce G_1 and G_2 checkpoint responses that can be attenuated by application of anti-oxidants (Clopton *et al.*, 1995, and Flattery-O'Brien *et al.*, 1998). It was suggested pATM mediates in both responses as a sensor of oxidative stress (Rotman *et al*., 1997) and that the inhibition of cyclin E/Cdk2 activity and induction of p53 play roles in the G_1 checkpoint arrest by ROS (Shackelford *et al.*, 2001).

ROS also affects the two induction pathways immediately upstream of the effector processes of apoptosis – the receptor-mediated death-signalling pathway and the mitochondrial pathway. Lethal levels of oxidative stress trigger apoptosis via the mitochondrial pathway. However, the generation of ROS is central to the progress through both induction pathways. ROS is generated during the Fas-mediated death receptor apoptosis pathway (Suzuki *et al.*, 1998) and over-expression of glutathione

peroxidase 1 appears to provide protection against it (Gouaze *et al*., 2002). Tumour necrosis factor (TNF)-induced caspase-3 activation, in the TNF-receptor death pathway, is suppressed by transfection of cells with superoxide dismutase (Kizaki *et al.*, 1993). Anti-oxidants, such as ascorbate, glutathione and other thiol reducing agents, prevent apoptosis via the mitochondrial pathway by regulating mitochondrial permeability transition (Custodo *et al.*, 2002), thereby preventing the release of cytochrome c and consequent activation of caspase 9 and 3. Anti-CD95 antibodies triggered an early generation of ROS in human breast cancer T47D cells that was blocked by overexpression of glutathione peroxidase 1 and inhibition of initiator caspase activation. Enforced expression of glutathione peroxidase also resulted in inhibition of CD95-induced effector caspase activation, DNA fragmentation, and apoptotic cell death (Gouaze *et al.*, 2002). In addition, it also prevents superoxide production after the release of cytochrome c from the mitochondria during the induction of apoptosis (Cai *et al.*, 1998). In an experiment involving a T-cell line, partial deactivation of GST-pi favors apoptosis (Bernardini *et al.*, 2000). These observations suggest that anti-oxidant enzymes play a critical role preventing the induction of apoptosis via both apoptotic pathways.

Oxidative stress experienced by a malignant cell is often greater than its benign counterpart. Because of genetic mutations and abnormal protein expression, the malignant cell experiences threats to cell survival. Activated macrophages release ROS, such as nitric oxide, to kill tumour cells (Cui *et al.*, 1994). T cells and macrophages release TNF against tumour cells, inducing apoptosis. Indeed, markers of oxidative stress have been reported to be higher in breast cancers compared to their surrounding normal breast tissue (Kumaragurupara *et al.*, 2002).

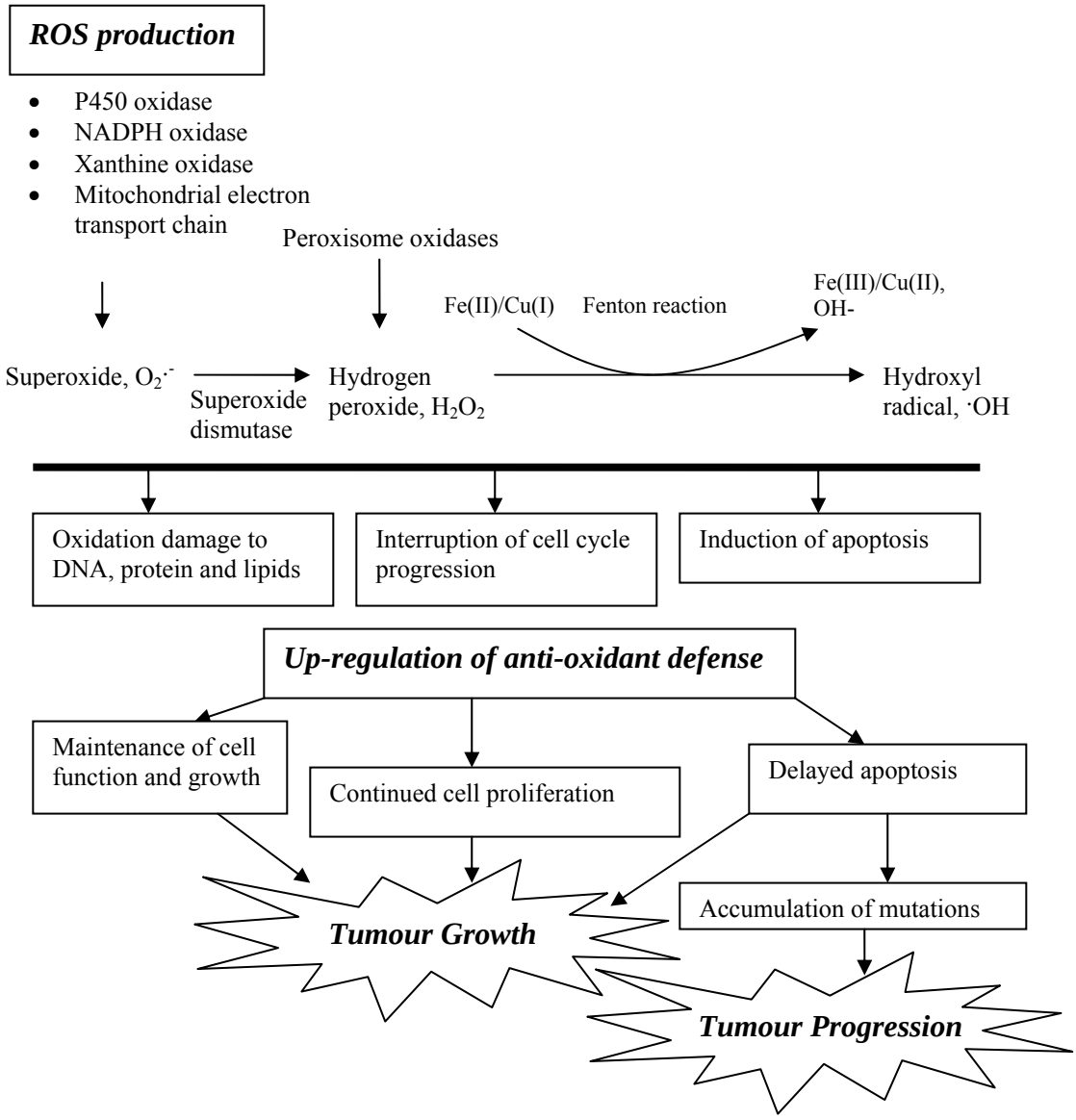


Figure 39. Up-regulation of anti-oxidant defences in the cancer cell protects it against the deleterious effects of ROS, allowing tumour growth and progression.

Furthermore, the intrinsic genetic mutations of the cancer cell exert a pressure for the termination of cell cycle and apoptosis. In fact, cancer cells are more susceptible to the inhibition of anti-oxidant enzymes compared to their normal counterparts, such that

inhibition of superoxide dismutase by certain estrogen derivatives selectively kill human leukemia cells, but not normal lymphocytes (Huang *et al.*, 2000).

Conversely, up-regulating cellular anti-oxidant defences allows cancer cells to survive longer, facilitating tumour progression (Figure 39).

Interestingly, the role of GST-pi in breast cancer extends beyond its ability to scavenge and ameliorate the effects of reactive oxidative species. GST-pi has been shown to inhibit the function of c-Jun N-terminal kinase (JNK) only recently.

JNK, also known as stress-activated MAP kinase (SAPK), is a group of proteins belonging to the mitogen activated protein kinase (MAPK) family. The signal cascade to JNK activation after a stress stimulus is not clear. What is known is that, a diverse group of MAPK kinase kinase (MAPKKK), such as ASK1, MEKK, MLK, TAK1 and TPL-2, is responsible for the subsequent activation of two known MAPK kinase (MAPKK) that activate JNK. These MAPKKs (MKK4 and MKK7) have been shown to be activated and accumulate in the nucleus in response to environmental stress, and these activate JNK in turn. JNK triggers apoptosis primarily through Bcl-2 and Bcl-xL phsophorylation, resulting in inhibition of their anti-apoptotic function (Davis, 2000).

In low stress states, it was shown that monomeric GST-pi binds to JNK and prevents it from interacting with its target proteins (Wang *et al.*, 2001). Oxidative stress causes the dissociation of the GST-pi/JNK complex and oligomerization of GST-pi; and addition of purified GST-pi caused a dose-dependent inhibition of JNK activity (Adler *et al.*, 1999).

Results showed that increased GST-pi expression, without considering other clinico-pathological factors, was associated with lower apoptotic index in breast cancers.

Further, there was no correlation between apoptotic index with GST activity in GST-pi negative tumours. In contrast, GST-pi positive tumours within the same range of oxidative stress showed a reduction in apoptosis with increased GST activity.

However, at higher levels of oxidative stress, GST activity in GST-pi positive tumours was no longer associated with reduced apoptosis. It is recognized that cellular response to extremes of oxidative stress experienced may not be dose-dependent (Halliwell, 2000). Probably the increase in GST activity in response to the higher oxidative stress was not sufficient to reduce apoptosis significantly.

4.3 GST-pi and Bcl-2

The possibility that GST-pi interacts with another apoptosis-related protein, Bcl-2 is raised in this study.

Bcl-2 is an important anti-apoptotic protein, originally identified by the translocation [t(14,18)] in follicular lymphomas. Induction of apoptosis by such external stimuli as radiation, hyperthermia, growth factor withdrawal, glucocorticoids and many classes of chemotherapeutic agents is inhibited by Bcl-2 in vitro.

Only 37% of the breast cancers exhibited Bcl-2 over-expression, compared with 49% (Sierra *et al*., 1998) and 63% (Le *et al.*, 1999) in two studies that used the same cutoff to define Bcl-2 over-expression. Whether such variations were due to population differences await further confirmatory studies. Nevertheless, consistent with those studies, a higher proportion of Bcl-2 over-expressing tumours remains associated with estrogen

receptor–positive tumours and tumours with better prognostic characteristics such as smaller size. A higher Bcl-2 immunoreactivity was also associated with a lower extent of apoptosis, a finding which is similar to that reported by Vakkala *et al.,* 1999.

Several mechanisms have been put forward to explain how Bcl-2 inhibits apoptosis. It has been suggested that Bcl-2 plays a part in regulating cellular redox potential in response to oxidative stress (Hockenbery, 1993). Bcl-2 is known to block lipid peroxidation and generation of reactive oxygen species and cellular redox potentials.

Moreover, Bcl-2 has been reported to alter intracellular ion fluxes that occur during apoptosis, including changes in the partitioning of Ca(II) in the cellular organelles such as endoplasmic reticulum, nucleus, and mitochondria (Marin, 1996). Bcl-2 has a C-terminal membrane anchor and is capable of forming ion channels in the mitochondria, as well as in the nucleus. The release of cytochrome c and apoptosis initiating factor from the mitochondria into the cytoplasm is an essential step in the induction of apoptosis via the mitochondrial pathway. Bcl-2 blocks mitochondrial permeability to these proteins (Yang *et al.*, 1997). On the nuclear membrane, Bcl-2 acts as a gate-keeper, regulating nuclear localization of p53 and NF-κB (Hermann *et al.*, 1996). More recently, Bcl-2 over-expression was shown to cause redistribution of glutathione from cytosol to the nucleus and glutathione depletion resulted in sensitization to apoptosis even in Bcl-2 over-expressing cells (Voehringer *et al.*, 1998). It has been suggested that nuclear glutathione plays a role in regulating transcription, through redox modification of the DNA binding regions of such proteins as p53, AP-1 and NF-κB (Sun *et al.*, 1996), which are important mediators of apoptosis.

There appeared to be an increased nuclear localization of the GST-pi protein in Bcl-2 overexpressing tumours, as clearly demonstrated by two-color immunofluorescence with confocal microscopy, whilst GST-pi immunoreactivity was observed to be diffusely located in the cytoplasm in most of the Bcl-2 negative tumours. Could GST-pi localization in the nucleus be mediated by Bcl-2 protein? GST-pi, which has a molecular weight of 22 kDa, has the potential to gain entry into the nucleus through the nuclear pore complex.

Coincidentally, nuclear expression of GST-pi has been reported under different circumstances. When cancer cells were exposed to doxorubicin and cisplatin, the cells which exhibited nuclear localization of GST-pi were resistant to apoptosis (Goto, 2001) and this protection was removed by application of an inhibitor of transport through the nuclear pore. GST-pi appeared to prevent the DNA damage caused by these cytotoxic drugs in *in vitro* experiments. In some way, localization of GST-pi in the nucleus protected against cell death.

The expression of Bcl-2 in cancers has been associated with increased mutation frequency (Cherbonnel-Lasserre *et al.*, 1996) and accumulation of oncogenes (Sierra *et al.*, 2000). By delaying cell death, the Bcl-2 protein may help to promote the accumulation of mutations, allowing cancer cells to acquire a more malignant phenotype. Other forms of apoptotic dysregulation are known to play an important role in breast cancer metastasis (Shin *et al.*, 2001).

However, Bcl-2 expression was not only not associated with increased lymph node metastasis, but was associated with significantly improved recurrence-free survival in the breast cancer patients. Indeed, Bcl-2 is known to be a favourable prognostic marker in

breast carcinoma (Fitzgibbons *et al.*, 2000). This raises the possibility that protection against apoptosis may not be sufficient explanation for the association of GST-pi expression with early recurrence.

4.4 GST-pi in association with MT expression

Whilst the significance of the association between GST-pi nuclear localization and Bcl-2 expression remains to be elucidated, other mechanisms may be operative to explain the association of GST-pi expression with aggressive breast cancers of high recurrence risk. It may be possible that GST-pi expression is associated incidentally with the concomitant dysregulation of a certain set of oncogenes in the breast cancers that result in an "aggressive phenotype"

This possibility has been suggested in early studies on GST-pi expression. Higher GST-pi expression was associated with reduced estrogen receptor level in the breast cancer (Howie *et al.*, 1988). This appears to be mediated by increased methylation of the GC box promoter of the GST-pi gene with estrogen receptor expression (Jhaveri *et al.*, 1998[b]) and decreased GST-pi mRNA stability in the estrogen receptor positive breast cancer cells (Jhaveri *et al.*, 1997).

The expression of metallothionein (MT), which has the potential to be of clinical importance in breast cancer, was investigated. Similar to GST-pi, MT is a protein marker in breast cancer that is associated with decreased estrogen receptor immunoreactivity when expressed, but (as in GST-pi) its immunopositive status is not necessarily associated

with estrogen receptor negative breast tumours (Oyama *et al.*, 1996). In contrast with GST-pi expression, MT immunopositivity (detected by antibody that binds to both MT-1 and MT-2 isoforms) is strongly associated with breast cancers of high histologic grade. This relation is present even when isoforms 2A and 1F are studied separately (Jin *et al.*, 2001 and 2002). Unfortunately, there was no correlation between GST-pi expression and MT immunoreactive score.

Metallothionein is a group of small metal-binding proteins (<7 kD) with 18-23 cysteine residues that bind metal ions, such as zinc, copper or cadmium in thiolate clusters. The major isoforms expressed in mammalian tissues are MT-1 and MT-2, and they are implicated in sequestration or storage of essential transition metal ions during processes of rapid cell proliferation such as fetal development and inflammation. They are also involved in the protection against metal toxicity (such as Cd), in protection against oxidative stress by binding to transition metals displaying Fenton reactivity (Fe and Cu), and possibly in protection against xenobiotics (Viarengo *et al.*, 2000).

Its involvement in processes of rapid cell growth and protection against oxidative stress suggests a role in carcinogenesis. This may be mediated through direct interaction with transcriptional activator, NFκB and modulation of p53, DNA and RNA polymerase activity by its effects on zinc homeostasis (Jin *et al.*, 2002). Down-regulation of metallothionein induces apoptosis and growth arrest in human breast cancer cells (Abdel-Mageed *et al.*, 1997). Increase in MT expression in human breast cancer tissues is primarily associated with an increase in proliferative markers. There was, however, no association between MT expression and apoptotic index.

Indeed, higher level of MT expression was associated with shorter disease-free survival and the 3 fold increase in risk of recurrence was independent of common clinicopathological factors (especially tumour grade) and GST-pi expression. The fact that this difference in disease-free survival was more apparent in the group of patients receiving adjuvant chemotherapy compared to the group which did not, suggests that MT plays a major role in protecting the cell from toxic insults (Kimura *et al.*, 2000).

Nuclear localization of MT is a more commonly reported phenomenon compared to that of GST-pi. It is believed that nuclear translocation of MT is an ATP-dependent process and the sub-cellular localization of MT is associated with the resistance to the toxicity of different metal containing compounds (Woo *et al.*, 1997). Interestingly, nuclear localization of MT is associated with improved disease-free survival, although the statistical significance of this association is only of marginal significance after taking into account the effects of the level of MT expression by multivariate analysis.

4.5 GST-pi and chemotherapy

The GST family of enzymes has long been implicated in chemotherapeutic drug resistance. Chemoresistance is a major cause of treatment failure, and modulation of cellular proteins such as GST-pi involved in detoxification has been suggested as one of the mechanisms that contribute to drug resistance (el-Deiry, 1997). Indeed, in human breast cancer tissues, GST-pi expressing tumours exhibited reduced cell death after neo-

adjuvant chemotherapy of 5-fluorouracil/doxorubin/mitomycin C compared to the GST-pi negative counterparts (Su *et al.*, 2003).

In this study, it is found that GST-pi expressing breast cancers had poorer disease-free survival after chemotherapy compared to the GST-pi negative tumours.

Of the common chemotherapeutic agents used as first-line treatment for breast cancer, cyclophosphamide is a known substrate of GST-pi and is inactivated to form glutathione s-conjugates. On the other hand, doxorubicin was found not to form similar conjugates in breast cancer cells selected for resistance against it (Gaudiano *et al*, 2000). In spite of this, it was observed that in many doxorubin resistance-selected breast cancer cell lines, GST-pi activity was consistently upregulated by as much as up to 45 times (Batist *et al.*, 1986, Whelan *et al.*, 1989, Gaudiano *et al*, 2000).

Interestingly, there is concomitant upregulation of GST-pi and p-glycoprotein (Pgp) in many of the breast cancer cell lines selected for resistance against doxorubicin (Whelan *et al.*, 1992). Parallel changes in Pgp and GST-pi expression was also observed in doxorubicin resistant breast cancer cells after exposure to nomegestrol (Li *et al.*, 2001). In fact, it was suggested in transfection studies that Pgp expression, not GST-pi, was the primary factor in determining resistance against chemotherapy (Fairchild *et al.*, 1990).

Pgp is one of several ATP-binding cassette transporters that are able to translocate multiple substrates across the cell membranes (Schwab *et al.*, 2003). It is distinct from the ATP dependent glutathione S-conjugate (GS-X) pump, the multi-drug resistance associated protein 1 (MRP1) and the multispecific organic anion transporter (MOAT) responsible for the transport of glutathione S-conjugates. Chemotherapeutic agents, anthracyclines (eg. doxorubicin and epirubicin) and taxanes (eg. paclitaxel and docetaxel),

are known substrates of Pgp and *in vitro* studies have shown that resistance to these drugs are associated with Pgp expression in breast cancer (Mechetner *et al.*, 1998).

The results of this study indicated that Pgp immunopositivity was associated with younger patients and estrogen receptor positive tumours, as well as GST-pi positive tumours. In fact, Pgp positivity was the only factor found to be significantly associated with GST-pi expression after multivariate analysis.

However, Pgp expression was not associated with worse disease-free survival after chemotherapy in the group of patients receiving adjuvant chemotherapy. Pooled studies of Pgp expression on the response of locally advanced breast cancer to neo-adjuvant chemotherapy showed an association with poorer complete response rate, but not overall response rate (Leonessa *et al.*, 2003). On the other hand, a study involving 85 node positive breast cancer patients receiving anthracycline-based adjuvant chemotherapy showed no difference in recurrence rates (Ferrero *et al.*, 2000).

Also studied, was the expression of Y-box binding protein-1 (YB-1), a protein that may be associated with Pgp expression.

YB-1 has been stated as "the most evolutionary conserved nucleic-acid-binding protein currently known" (Kohno *et al*., 2003). YB-1 belongs to a group of DNA and RNA binding proteins that has a conserved cold shock domain which interacts with inverted CCAAT boxes (Y-boxes) (Izumi *et al.*, 1991). It is found to regulate gene expression through both transcription and translation (Matsumoto *et al*, 1998). Hence, this protein is believed to play an important role in the cell cycle (Jurchott *et al.*, 2003). YB-1 has also been linked to a number of cellular responses to stress and carcinogenic stimuli. It has been reported to have an affinity for depurinated and cisplatin modified DNA

(Boulikas, 1996 and Ise *et al.*, 1999), as well as RNA damaged by reactive oxygen species (Hayakawa *et al*, 2002). YB-1 has been found to translocate to the nucleus when the cell is exposed to UV irradiation (Koike *et al.*, 1997) and heat (Stein *et al.*, 2001) and is involved in redox-dependent transcription activation (Duh *et al.*, 1995). In addition, YB-1 is known to be up-regulated in cell-lines which are resistant to genotoxic agents (Levenson *et al.*, 2000 and Ohga *et al.*, 1996).

The role of YB-1 in cancer progression has attracted attention in recent years. Increased YB-1 expression has been correlated with DNA topoisomerase IIα and proliferating cell nuclear antigen expression in human lung cancer (Gu *et al.*, 2001) and colorectal cancer (Shibao *et al.*, 1999) and linked to markers of cellular proliferation in osteosarcoma (Oda *et al.*, 1998). In addition, YB-1 is thought to promote metastasis by promoting the transcription of gelatinase A, a matrix proteinase that facilitates cell migration (Cheng *et al.*, 2002). Moreover, expression of YB-1 protein has been reported to reflect chemosensitivity of ovarian serous adenocarcinoma (Kamura *et al.*, 1999). In this context, YB-1 expression has also been shown to be associated with Pgp expression in breast cancer cells, resulting in multi-drug resistance (Bargou *et al.*, 1997).

In this study, two different antibodies to detect the expression of YB-1 in breast cancers were used. The immunoreactive scores obtained for each of the antibodies correlated well and the conclusions obtained from the results were similar.

The results show that high YB-1 expression was associated with breast cancers of an aggressive phenotype (lymph node positive/hormone receptor negative). And differences in YB-1 expression resulted in a measurable effect on the clinical course of breast cancer. Without chemotherapy, primary breast cancers with higher YB-1

expression have a higher recurrent risk compared to those with lower expression. This result is consistent with a separate study involving primarily Caucasian breast cancer patients (Janz *et al.*, 2002). Interestingly, GST-pi positive tumours tended to have a higher YB-1 scores but statistical significance for the association was lost on multivariate analysis with other pathological factors.

The significance of nuclear localization of YB-1 in breast cancer is yet unclear. Nuclear localization of YB-1 was found to affect prognosis in squamous cell lung cancer, ovarian serous adenocarcinoma and synovial sarcoma, but not in lung adenocarcinoma (Shibahara *et al*., 2001; Oda *et al.*, 2003; and Yahata *et al.*, 2002). It has also been reported that nuclear localization of the YB-1 protein was found to be associated with high levels of Pgp expression in human breast cancer (Bargou *et al*, 1997) and osteosarcoma (Oda *et al.*, 1998), but not in lung (Gu *et al.*, 2001) and colorectal cancer (Shibao *et al.*, 1999). Similarly, this study showed that nuclear localization of YB-1 is associated with a propensity for Pgp expression. However, the proportion of breast cancers with nuclear localization of YB-1 appears to be low as evidenced by 11% in this present study and 13% of 86 patients in that reported by Janz *et al.* (2002) as compared to other cancers, such as non-small cell lung cancer (45% of 196 patients) (Oda *et al.*, 2003) and ovarian cancer (45% of 35 patients) (Yahata *et al.*, 2002).

Using a computational approach based on the Resonant Recognition Model, it is also shown that direct binding between the YB-1 protein and the MDR1 gene promoter is indeed possible, further verifying experimental evidence that YB-1 is involved in the regulation of MDR1 gene in cancer cells (Bargou *et al.*, 1997 and Ohga *et al.*, 1998). However, the present study also shows that the absence of nuclear localization of YB-1

does not necessarily preclude Pgp expression. In those tumour samples, it is possible that nuclear localization of YB-1 may not have been prominent enough to be detected by immunohistochemistry, or that other promoters are also operative (Hu *et al.*, 2000).

The possible effect of YB-1 on Pgp expression and the association between the level of YB-1 expression and GST-pi positivity in breast cancers suggest that YB-1 may confer multi-drug resistance in breast cancer. In addition, YB-1 may affect chemotherapy resistance in many other ways, possibly through DNA repair (Izumi *et al.*, 1991), or transcriptional or translational control of other proteins conferring drug resistance.

Taking all the patients receiving chemotherapy as a group, there was no statistically significant difference in recurrence risk between patients with low YB-1 expression and higher YB-1 expression who received adjuvant chemotherapy. However, it is interesting that although most of the patients received CMF chemotherapy, 80% of those patients who suffered tumour recurrence and had YB-1 scores of 5 or more, received anthracycline-based (eg. doxorubicin) chemotherapy regimes. This suggests that YB-1 expression in breast cancer may possibly predict tumour resistance to different chemotherapy regimes, providing a marker that aids in the choice of adjuvant chemotherapy for breast cancers. As CMF and anthracycline-based regimes are mainstays of breast chemotherapy, larger clinical studies will be useful to verify if YB-1 expression would be useful in determining the choice of chemotherapeutic regimes for breast cancer patients.

4.6 Conclusion

GST-pi appears to be a promising prognostic marker for early breast cancers. In this study, it was found that:

1. GST-pi may have a role in ameliorating the effects of oxidative stress on apoptosis;
2. Nuclear localization of GST-pi is associated with Bcl-2 expression;
3. GST-pi expression in the tumours, together with Bcl-2 expression and low tumour grade, is associated with lower apoptotic index, but the effect was not statistically significant if all clinicopathological factors are considered in multivariate analysis;
4. Amongst the biological markers examined with GST-pi, MT expression is not associated with GST-pi expression, but is associated with high grade tumours.
5. On the other hand, YB-1 and Pgp expression are associated with GST-pi expression, but only the association with Pgp expression remained statistically significant by multivariate analysis;
6. GST-pi expression is associated with poorer disease-free survival; and
7. GST-pi expression is also associated with poorer prognosis in the group of patients who received adjuvant chemotherapy, whilst difference in disease-free survival was not seen in this group of patients when stratified according to Pgp or YB-1 expression.

Indeed, GST-pi expression is associated with more aggressive tumours and this effect may be partly explained by protection against oxidative stress and apoptosis, supporting our initial hypothesis.

At the same time, the study developed further understanding with regards to the clinical importance of several other tumour markers:

1. Bcl-2 expression, although associated with increased apoptosis, was associated with estrogen receptor positive and smaller sized tumours, and is an independent favourable prognostic factor;
2. Nuclear expression of MT is associated with lower recurrence risk;
3. High level of MT expression is associated with poorer disease-free survival and this effect is more evident in the group of patients who received adjuvant chemotherapy;
4. High level of YB-1 expression is associated with poorer disease-free survival in patients who did not receive adjuvant chemotherapy;
5. High level of YB-1 expression is associated with recurrence in patients who have received anthracycline-based chemotherapy; whilst lower levels of YB-1 expression is associated with recurrence in patients who were given the CMF regime.

4.7 Future studies

This study has provided insights into the biological role of GST-pi in breast cancer and the clinical importance of GST-pi, MT, Pgp and YB-1 as potential prognostic markers for early recurrence. The observations regarding the nuclear localization of GST-pi and MT

could be further explored by studies examining how sub-cellular localization affects gene expression, possibly through the use of micro-array technology.

With recent interest in pharmacogenetics, several polymorphisms of the GST-pi gene have been identified with different stability and varying activity towards electrophilic substrates (Lin *et al.*, 2003). These polymorphisms have different allelic frequencies in different racial groups. For example, the allelic frequency for the less active Ile105Val variant is 0.43 for whites, 0.28 for African-Americans and 0.17 in a Chinese population (Wang *et al*., 2003). This may explain the varying response to chemotherapy amongst patients (Sweeney *et al.*, 2000). Of further interest will be how these polymorphisms modify the phenotype of breast cancer in the subgroup of breast cancer patients who do not receive adjuvant chemotherapy.

The high frequency of GST-pi expression in breast cancer and its strong association with recurrence risk suggests it as a likely therapeutic target. Apoptosis-based anticancer drugs are an attractive area of development as they are potentially selective against cancer cells (Zhang, 2002). Cancer cells, under higher apoptotic stress compared to normal cells, are more sensitive to perturbation to part of their apoptotic defence.

In addition, as more patients with early stage breast cancers are recommended chemotherapy, the usefulness of GST-pi, MT and YB-1 in identifying patients at higher risk of recurrence after chemotherapy is promising. Of particular interest is YB-1 expression. Larger clinical studies will be needed to verify if YB-1 expression could help in determining the choice of chemotherapeutic regimes for breast cancer patients.

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Appendix I

LIST OF PATIENTS

	patient no.	age (years)	Tumour size (cm)	grade	nodal status	estrogen receptor	adjuvant chemotherapy	days to recurrence
1	1072	40	4.0	3	Pos	Neg	No	262
2	1146	45	3.0	3	Pos	Pos	Yes	
3	1518	58	6.0	3	Pos	Neg	Yes	523
4	1558	61	2.0	3	Neg	Pos	Unk	Unk
5	1579	33	2.0	3	Pos	Pos	Yes	
6	1583	49	1.6	3	Neg	Pos	No	
7	1584	79	3.0	2	Neg	Pos	No	694
8	1588	40	1.0	2	Neg	Pos	No	
9	1937	72	4.0	3	Neg	Pos	No	
10	1995	39	2.8	2	Pos	Neg	Yes	
11	2102	58	1.4	2	Neg	Pos	No	
12	2242	53	3	3	Pos	Neg	Unk	Unk
13	2324	65	5	1	Pos	Pos	Unk	Unk
14	2684	63	3	2	Neg	Pos	Unk	Unk
15	2703	85	4.5	3	Pos	Neg	Unk	Unk
16	2763	47	2.5	2	Neg	Pos	Unk	Unk
17	2832	49	6	3	Pos	Neg	Unk	Unk
18	3117	49	2.0	3	Pos	Pos	Yes	
19	3143	64	2.8	2	Pos	Pos	No	
20	3204	58	4.0	1	Pos	Pos	No	
21	3347	76	2.5	3	Neg	Neg	No	
22	3528	51	2.5	2	Pos	Pos	Yes	
23	4412	44	3.5	3	Neg	Pos	Yes	
24	4430	33	2.0	2	Pos	Pos	Yes	
25	4434	44	4.0	1	Neg	Pos	Yes	
26	4644	47	3.5	1	Pos	Pos	Yes	
27	4746	53	1.4	2	Pos	Neg	Unk	Unk
28	4794	52	3.5	3	Neg	Pos	Yes	
29	4795	67	4.0	2	Pos	Pos	No	
30	5088	64	4.5	1	Pos	Pos	Yes	
31	5168	47	1.3	3	Pos	Neg	Yes	
32	5204	62	5.0	2	Neg	Neg	Yes	678
33	5339	59	3.0	2	Neg	Pos	No	
34	5353	50	3.0	2	Neg	Pos	Yes	
35	5482	44	6.0	3	Pos	Pos	Yes	
36	5535	43	2.2	3	Neg	Neg	No	
37	5797	49	3.4	2	Pos	Pos	No	
38	5820	86	5.0	3	Pos	Neg	No	
39	6114	52	2.0	3	Neg	Neg	Yes	
40	6210	41	2.0	3	Neg	Neg	Yes	
41	6218	40	4.5	3	Pos	Pos	Yes	
42	6471	44	3.0	2	Pos	Pos	Yes	
43	6473	68	3.0	2	Pos	Pos	No	
44	6683	72	4.5	3	Neg	Pos	No	

45	6794	58	7.0	2	Neg	Pos	Yes	
46	6834	52	4.5	3	Pos	Pos	No	
47	6964	59	3.3	3	Pos	Pos	Yes	
48	7031	51	1.7	3	Neg	Pos	Yes	
49	7151	51	3.0	3	Pos	Pos	No	
50	7179	42	3.0	2	Pos	Pos	Yes	
51	7423	63	6.0	3	Pos	Neg	No	
52	7425	46	2.3	1	Pos	Pos	Yes	
53	7440	34	4.5	3	Neg	Neg	Yes	
54	7838	56	2.5	3	Pos	Pos	No	1206
55	7839	57	3.5	2	Neg	Pos	Yes	
56	7840	35	2.3	3	Pos	Neg	Yes	489
57	7847	48	2.2	2	Pos	Pos	Yes	
58	8097	54	3.0	3	Unk	Neg	No	
59	8183	47	3.5	3	Neg	Pos	Yes	
60	8461	71	2.5	3	Neg	Pos	Unk	Unk
61	9164	59	4.5	3	Neg	Pos	No	717
62	9167	64	3.5	2	Neg	Neg	Yes	
63	9786	56	3.0	1	Pos	Pos	Yes	
64	11042	55	2.8	1	Pos	Pos		
65	11046	66	4.5	3	Neg	Neg	Unk	Unk
66	11898	58	1.5	2	Neg	Pos	Unk	Unk
67	12337	64	4.5	3	Pos	Pos	Unk	Unk
68	13482	81	2.5	3	Neg	Neg	No	
69	13714	46	7.5	2	Pos	Neg	No	646
70	13750	57	3.5	3	Neg	Neg	Yes	
71	16016	41	2.0	3	Neg	Neg	Yes	
72	16048	58	5.0	3	Pos	Neg	Yes	458
73	16067	48	2.7	3	Pos	Pos	Yes	
74	16068	55	3.0	3	Pos	Pos	Yes	
75	16343	48	2.5	2	Neg	Pos	Yes	
76	16494	49	2.2	3	Neg	Pos	Yes	
77	16934	49	6.0	2	Pos	Pos	Yes	649
78	16999	65	4.5	2	Pos	Neg	Yes	
79	17621	58	2.5	3	Neg	Pos	No	
80	17626	43	4.5	3	Neg	Neg	Yes	
81	17813	52	1.8	2	Neg	Pos	No	
82	18074	57	3.0	3	Pos	Neg	Yes	
83	19001	56	3.5	2	Pos	Neg	Yes	1055
84	19116	78	5.0	2	Neg	Neg	Yes	
85	19128	38	4.7	2	Pos	Neg	Yes	
86	19420	49	1.3	1	Pos	Pos	Yes	
87	19486	60	2.5	3	Pos	Neg	Yes	
88	19488	35	4.5	3	Pos	Neg	Yes	
89	20067	80	11.0	3	Pos	Neg	No	
90	20669	61	5.0	2	Pos	Neg	Yes	
91	21345	52	2.0	2	Pos	Neg	Yes	
92	21357	64	4.0	3	Pos	Pos	Yes	
93	21752	55	2.1	2	Pos	Pos	Unk	Unk
94	21780	51	2.5	3	Neg	Pos	Yes	

95	21796	54	4.8	3	Pos	Pos	Unk	Unk
96	21936	45	6.0	3	Pos	Pos	Yes	371
97	22003	57	2.0	2	Pos	Pos	No	
98	23103	70	12.0	2	Pos	Neg	No	
99	23233	55	4.0	3	Pos	Neg	Yes	
100	23341	46	3.0	2	Pos	Unk	Yes	
101	23383	56	3.5	3	Neg	Pos	No	
102	23471	36	2.0	3	Unk	Pos	Yes	798
103	23768	59	4.5	3	Neg	Neg	Yes	1116
104	24025	75	4.5	3	Pos	Pos	No	282
105	25286	33	4.0	3	Pos	Neg	Yes	419
106	25398	48	5.0	2	Pos	Pos	Yes	
107	25400	55	3.0	3	Neg	Neg	Yes	
108	25841	44	2.0	3	Neg	Unk	No	
109	26974	40	6.0	2	Pos	Pos	Yes	
110	26976	64	3.0	1	Pos	Pos	No	
111	27230	45	2	3	Neg	Neg	Unk	Unk
112	27642	77	3.0	3	Neg	Neg	No	
113	27839	57	1.8	2	Neg	Pos	No	
114	27840	77	3.0	1	Pos	Pos	No	
115	27844	61	1.4	2	Neg	Neg	No	
116	27919	63	2.2	1	Neg	Pos	No	
117	27952	56	1.2	2	Neg	Pos	Unk	Unk
118	28305	64	3.0	2	Neg	Pos	No	883
119	28954	68	3.0	3	Pos	Neg	Yes	
120	30071	35	2.5	2	Neg	Pos	Yes	750
121	30388	38	2.0	3	Pos	Pos	Yes	
122	30531	48	2.0	2	Pos	Pos	No	
123	30640	53	3.5	3	Neg	Neg	Unk	Unk
124	30642	49	4.8	3	Pos	Neg	Unk	Unk
125	31077	75	7.0	3	Pos	Neg	No	603
126	31082	79	3.0	3	Pos	Neg	No	747
127	31148	51	2.5	1	Neg	Pos	Yes	
128	31152	46	4.5	2	Neg	Pos	Yes	
129	31153	38	2.8	3	Neg	Pos	Yes	
130	31333	73	3.0	3	Neg	Pos	No	
131	31366	58	2.1	2	Neg	Pos	No	
132	31389	79	4.0	3	Neg	Pos	No	
133	31467	43	4.0	3	Neg	Pos	Yes	
134	31674	48	8.5	2	Pos	Pos	Yes	367
135	32453	46	3.5	2	Neg	Pos	Yes	
136	34349	60	2.4	2	Pos	Pos	Unk	Unk
137	34435	47	2.9	3	Neg	Pos	Unk	Unk

Neg = negative; Pos = positive; Unk = unknown

Appendix II

SOLUTIONS AND REAGENTS

APES coated slides

Acid washed slides coated with 2% 3-aminopropyl-tri-ethoxysilane (Sigma-3648) in acetone

Citrate buffer

0.01M sodium citrate adjusted to pH 6 by 0.01M citrate acid.

Diaminobenzidine (DAB) reaction solution

3.3'-diaminobenzidine tetrachloride	50mg
Tris buffer or Tris buffered saline	100ml
H_2O_2	33µl

Eosin staining solution

1% Eosin in deionised H_2O

Harris Haematoxylin

Haematoxylin	1g
Absolute alcohol	10ml
Ammonium alum (or potassium alum)	20g
Mercuric oxide	0.5g
Deionised H_2O	200ml

Lysis buffer

10mM Tris, pH 7.4

1% SDS (sodium dodecyl sulfate)

Methyl green staining solution

0.5% methyl green in 0.1M acetate buffer pH 4.8

50mM Phosphate buffer pH 7.4

K_2HPO_4	(0.87g/100ml)	74ml
NaH_2PO_4	(0.6g/100ml)	27ml

0.1M Phosphate buffer pH 6.5

K_2HPO_4	(1.74g/100ml)	30ml
NaH_2PO_4	(1.2g/100ml)	78ml

50mM Phosphate buffered saline (PBS)

$Na_2HPO_4.7H_2O$	13.4g
NaCl	8g
H_2O	made up to 1L

Adjusted to pH 7.4 with dilute HCl

50mM Tris buffer (TB) pH7.4

tris(Hydroxymethyl)aminomethane	6g
H_2O	made up to 1L

Adjusted to pH 7.4 with dilute HCl

50mM Tris buffered saline (TBS) pH7.4

tris(Hydroxymethyl)aminomethane	6g
NaCl	8g
H_2O	made up to 1L

Adjusted to pH 7.4 with dilute HCl

TBST buffer

10mM Tris pH8.0

100mM NaCl

1% Tween-20

Appendix III

Prognostic Significance of Glutathione *S*-Transferase-Pi in Invasive Breast Cancer

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Glutathione *S*-transferase pi (GST-pi), a Phase II detoxification enzyme, has recently been implicated in protection against apoptosis. Expression of GST-pi and Bcl-2 protein, an established apoptosis marker, was analyzed by immunohistochemistry in 116 cases of infiltrative ductal breast carcinomas in Singapore women. The markers were correlated with apoptosis detected by the TUNEL method and clinico-pathological parameters. There were 67 (58%) GST-pi–positive breast tumors and 43 (37%) Bcl-2–positive tumors. In a large proportion of GST-pi–positive/Bcl-2–positive tumors, there was a distinct accumulation of the GST-pi enzyme within the nucleus of cancer cells when examined by double immunofluorescence labeling under confocal microscopy. GST-pi immunoreactivity was not significantly correlated with any of the traditional histologic factors known to influence prognosis, whereas Bcl-2 overexpression was associated with reduced size of primary tumor ($P = .021$) and positive estrogen receptor status ($P = .001$). Univariate analysis revealed that GST-pi–positive, Bcl-2–positive, and lower histological grade tumors had decreased levels of apoptosis ($P = .024$, $P = .011$, and $P = .029$, respectively). However, multivariate analysis showed that histological grade and Bcl-2, but not GST-pi, immunoreactivity were correlated with apoptotic status. The Kaplan-Meier disease-free survival curves showed a significant difference between GST-pi–positive and GST-pi–negative breast cancer cases ($P = .002$). Disease-free survival in patients with GST-pi–positive tumors was also worse than that in patients with GST-pi–negative tumors in the group who had adjuvant chemotherapy ($P = .04$). In patients who were lymph node positive, GST-pi immunopositivity was found to influence disease-free survival. Recurrence of tumors was also significantly affected by GST-pi immunoreactivity (relative risk of 8.1). The findings indicate that GST-pi–positive tumors are more aggressive and have a poorer prognosis than do corresponding GST-pi–negative breast cancers.

KEY WORDS: Apoptosis, Bcl-2, Breast carcinoma, Confocal microscopy, Glutathione *S*-transferase, Immunohistochemistry, Survival.

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Glutathione *S*-transferase-pi (GST-pi) belongs to a major group of detoxification enzymes that is widely distributed in the human body (1). In normal human tissues, the enzyme protects cells against noxious compounds by catalyzing conjugating reactions with glutathione and protects against reactive oxygen species by reducing organic hydroperoxides via glutathione peroxidase activity (2). The role of GST-pi in tumor growth and progression is less well known. It is reported to be variably expressed in breast cancer (3) and is associated with estrogen receptor level expressed by the tumor (4, 5). Down-regulation of GST-pi activity in a T-cell line study appears to favor apoptosis (6) and inhibition of GST-pi function induces apoptosis in rat hepatoma cells (7).

The Bcl-2 protein is known to block apoptosis and prolong the life span of cells (8). It has been suggested that Bcl-2 plays a part in regulating cellular redox potential in response to oxidative stress (9). Bcl-2 is known to block lipid peroxidation and generation of reactive oxygen species and cellular redox potentials. Moreover, Bcl-2 has been reported to alter intracellular ion fluxes that occur during apoptosis, including changes in the partitioning of Ca^{2+} in the cellular organelles such as

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endoplasmic reticulum, nucleus, and mitochondria (10). The ability of the Bcl-2 protein to suppress apoptosis has also been linked with glutathione (11).

In the present study, the expression of GST-pi was analyzed by immunohistochemistry in breast cancer in relationship with Bcl-2 expression, apoptosis, and clinical outcome. The aim of this study was to determine the prognostic roles of GST-pi and Bcl-2 in patients with invasive ductal breast cancer. The results were correlated with clinicopathological parameters and disease-free survival.

MATERIAL AND METHODS

Patients, Tumors, Pathological Evaluation, and Clinical Follow-Up

The study population comprised 116 women diagnosed with infiltrative ductal breast carcinomas who had undergone mastectomy or lumpectomy without neoadjuvant treatment between 1998 and 1999 in the Singapore General Hospital. The tissues were fixed in 10% buffered formaldehyde (pH 7.0). The age of the patients ranged from 33 to 86 years, with a median age of 52 years. There was no distant metastasis at the time of diagnosis. The tumor size, estrogen receptor status, and axillary lymph node positivity were determined. Histologic grade of the tumors was established according to the criteria described by Sloane *et al.* (12). Information was not available on the estrogen receptor status of two patients because they were not analyzed at the time of diagnosis. Lymph node status in two patients was unknown because they did not undergo axillary lymph node dissection. Seventy-one (61%) patients received cytotoxic chemotherapy in addition to surgical removal of the tumors. Thirty-six (51%) patients received the cyclophosphamide/methotrexate/5-fluorouracil (CMF) regime, whereas the rest received adriamycin-based regimes such as adriamycin/cyclophosphamide, adriamycin/taxol, and cyclophosphamide/adriamycin/5-fluorouracil. Patient follow-up ranged from 33 to 1464 days, with a median follow-up of 1117 days. Twenty-one patients suffered recurrences, and mean disease-free interval was 981 days.

Immunohistochemistry

Paraffin-embedded sections were stained immunohistochemically for GST-pi using the polyclonal antibody anti-GST-pi antibody (Dako) at 1:200 dilution as described in detail previously (13). For Bcl-2 expression, sections were preheated in 10 mmol/L citrate buffer at pH 6.0 before incubation with a mouse monoclonal to human Bcl-2 (Cymbus Biotechnology) at 1:20 dilution for 90 minutes. After washing and incubation with the appropriate secondary antibodies, avidin-biotin-peroxidase complex was applied for 1 hour at room temperature to amplify the specific binding of primary antibody. Visualization was achieved by incubating with 3,3' diaminobenzidine tetrachloride (Sigma) as the peroxidase substrate. The sections were then counterstained with hematoxylin. GST-pi expression was considered to be positive when >10% of tumor cells exhibited cytoplasmic or nuclear staining. Bcl-2 expression was classified as positive when >30% of tumor cells exhibited cytoplasmic staining (14).

Double Immunofluorescence Labeling

Co-localization of GST-pi and Bcl-2 was performed on paraffin-embedded samples as previously described (15). The primary antibodies were incubated in the same medium with the tissue sections at the same concentrations as when they were used singly for immunohistochemistry. Cy3-conjugated secondary anti-rabbit antibody (to detect the GST-pi antibody) at a dilution of 1:800 and FITC-conjugated secondary anti-mouse antibody (to detect the Bcl-2 antibody) at a dilution of 1:200 were then applied for 1 hour at room temperature. After washing in PBS, the sections were mounted with fluorescence mounting media (DAKO). Stained sections were viewed and photographed using the LSM 510 Carl Zeiss confocal laser scanning microscope (equipped with an argon laser) under a Plan Apo 63 × 1.4 NA (oil) objective. Excitation wavelength for Cy3 was at 543 nm, and for FITC, at 488 nm.

TUNEL Method

For the detection of apoptosis in tissue sections, DNA fragmentation was identified using the terminal deoxynucleotidyl transferase–mediated, dUTP-biotin nick end-labeling (TUNEL) technique with the commercially available TdT-FragELTM DNA Fragmentation Detection kit (Oncogene Research Products) (16) in 113 tumor sections. After deparaffinization and rehydration, slides were permeabilized in 20 mg/mL of proteinase K. Endogenous peroxidase was inactivated by treating with 3% hydrogen peroxide. Subsequently, the sections were end-labeled with biotinylated dNTP by TdT for 2 hours at 37° C, and labeled cells were detected using streptavidin-horseradish peroxidase conjugate followed by diaminobenzidine staining. The apoptotic index was defined as the number of apoptotic nuclei per 100 cancer cell nuclei.

Statistical Analysis

For statistical analysis, SPSS software for Windows, Release 10.0, was used. Clinicopathological data was compared between groups of breast tu-

mors with different protein expression, using either χ^2 or Fisher's exact test. The Student's t test was used to compare the mean apoptotic index for different groups of breast tumors. For multivariate analysis of factors associated with apoptosis, log transformation and multiple linear regression was used. Survival curves were plotted by the Kaplan-Meier method, and the differences between the curves were evaluated by log-rank test. To assess the factors influencing recurrence, multivariate analysis using Cox's proportional hazard models was performed. A P value of $<.05$ was considered significant.

RESULTS

There were 67 (58%) GST-pi–positive breast tumors with the GST-pi protein being expressed in the cytoplasm, the nucleus, or both (Fig. 1A). Forty-three (37%) tumors were found to be bcl-2 positive (Fig. 1B). Thirty-three (28%) tumors were both Bcl-2 and GST-pi negative, and 27 (23%) were both Bcl-2 and GST-pi positive. Although there was no correlation between GST-pi and Bcl-2 expression (P = .441), Bcl-2 expression appeared to affect the localization of GST-pi. In a large proportion of GST-pi–positive/Bcl-2–positive tumors examined, there was a distinct accumulation of the GST-pi enzyme within the nuclei of cancer cells in addition to the cytoplasmic staining when observed by double immunofluorescence labeling under confocal microscopy (Fig. 1, C–E). Localization of Bcl-2 in the cytoplasm is represented by green fluorescence, and GST-pi expression in the cytoplasm/nucleus, by red fluorescence, whereas co-localization of cytoplasmic GST-pi with Bcl-2 is shown by orange fluorescence.

Table 1 shows the distribution of patients according to clinical and histopathological variables. The proportion of GST-pi–positive tumors was not significantly correlated with any of the histologic factors traditionally used for prognosis. In contrast, Bcl-2 overexpression was associated with reduced size of primary tumor (P = .021) and positive estrogen receptor status (P = .001).

Interestingly, GST-pi–positive tumors and Bcl-2–positive tumors had significantly lower apoptotic indices compared with the case of their negative counterparts (Table 2). Histological Grade I and II tumors also had lower apoptotic indices as com-

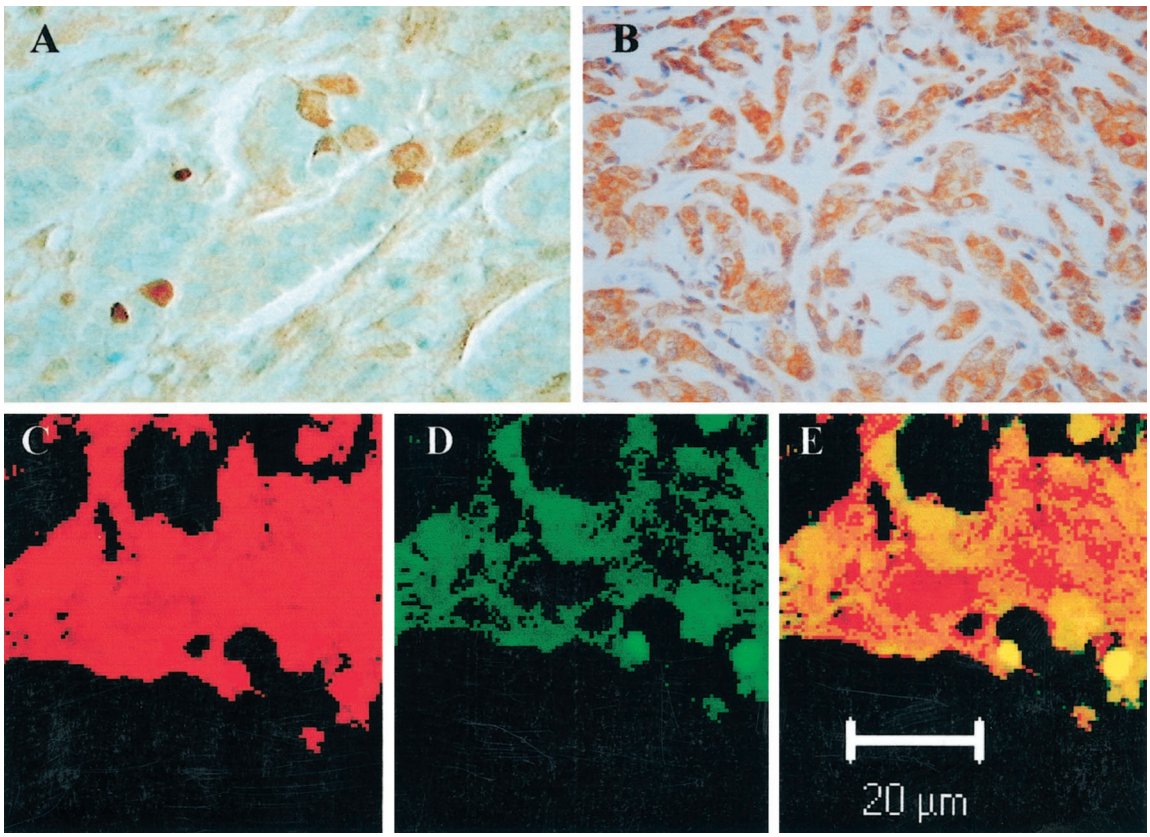

FIGURE 1. Composite figures of immunohistochemical stains of GST-pi (**A**) and Bcl-2 (**B**) and double immunofluorescence staining of GST-pi (**C–E**). **A,** cytoplasmic staining of GST-pi protein in a few breast cancer cells. Immunoperoxidase stain; original magnification, 200×. **B,** Bcl-2–positive immunoreactivity in breast cancer showing cytoplasmic staining. Immunoperoxidase stain; original magnification, 400×. **C,** breast cancer cells staining positively with **red fluorescence** for GST-pi immunoreactivity. **D,** breast cancer cells staining positively with **green fluorescence** for bcl-2 immunoreactivity. **E**, co-localization of GST-pi and bcl-2 in the cytoplasm of the breast cancer cells (**orange fluorescence**). Nuclear localization of GST-pi is indicated by the **red fluorescence**. (**C, D,** and **E,** bar = 20 μm).

TABLE 1. Relation between Over-Expression of GST-pi and Bcl-2 with Clinico-Pathological Factors

Clinico-Pathological Factors	Number of Patients	GST pi-Positive	Bcl2-Positive
Patient age			
50 years and below	50	31	23
More than 50 years	66	36	20
P		.453	.120
Primary tumor			
T1	21	12	13
T2	82	47	26
T3 and T4	13	8	3
P		.613	.021
Regional Lymph nodes			
Positive	63	35	21
Negative	51	30	21
P		1.000	.560
Estrogen receptor			
Positive	74	44	35
Negative	40	23	6
P		.845	.001
Histologic grade			
I	11	7	6
II	43	24	16
III	62	36	21
P		.894	.425

TABLE 2. Mean Apoptotic Index in Relation to Clinico-Pathological Factors

Clinico-Pathological Factors	Apoptotic Index (Mean ± SEM)
Patient age	
50 years and below	1.22 ± 0.22
More than 50 years	1.40 ± 0.20
P	.547
Primary tumor	
T1	0.94 ± 0.29
T2, T3 and T4	1.40 ± 0.17
P	.236
Regional lymph nodes	
Positive	1.42 ± 0.22
Negative	1.21 ± 0.20
P	.439
Estrogen receptor	
Positive	1.27 ± 0.21
Negative	1.49 ± 0.20
P	.486
Histologic grade	
I and II	0.97 ± 0.17
III	1.62 ± 0.23
P	.029
GST-pi expression	
Positive	1.05 ± 0.14
Negative	1.72 ± 0.30
P	.024
Bcl-2 expression	
Positive	0.82 ± 0.12
Negative	1.60 ± 0.22
P	.011

pared with the case of the Grade III tumors. There was no correlation of the apoptotic index with tumor size, axillary lymph node positivity, or estrogen receptor status. However, when analyzed by multivariate analysis, only histological grade and Bcl-2 immunoreactivity were found to be correlated with apoptosis ($P = .008$ and $P = .015$, respectively), whereas there was no association between GST-pi immunoreactivity and apoptosis ($P = .177$).

The Kaplan-Meier survival curves revealed a significant difference between GST-pi–positive and GST-pi–negative cases ($P = .002$; Fig. 2) but not between Bcl-2–positive and Bcl-2 negative ones ($P = .08$; Fig. 3). The time to recurrence was also significantly affected by GST-pi immunoreactivity ($P = .007$), with GST-pi immunopositive tumors having a shorter recurrence-free interval, whereas there was no association with Bcl-2 immunoreactivity ($P = .09$). Surgically treated breast cancer patients who received adjuvant chemotherapy and are categorized by GST-pi and Bcl-2 status are shown in Table 3. Although there was no significant difference in disease-free survival between Bcl-2 status and the treatment regime after surgery ($P = .14$ and $P = .21$ for surgery alone and surgery with adjuvant chemotherapy respectively), GST-pi–positive cases had significantly poorer survival in both groups of patients (Fig. 4 and Fig. 5).

Even though the mean number of positive axillary lymph nodes in GST-pi–positive and GST-pi–negative cases was not significantly different (4.0 ± 0.7 *versus* 6.0 ± 1.1 respectively; $P = .134$), GST-pi immunoreactivity was observed to influence disease-free survival in lymph node–positive cases. ($P = .002$; Fig. 6). Details of this subset of patients with respect to other clinicopathologic parameters are shown in Table 4.

When factors influencing recurrence were analyzed by multivariate analysis, GST-pi status was found to significantly influence disease-free survival ($P = .006$), with Bcl-2 status having a marginal influence over disease-free survival ($P = .067$; Table 5). The risk of tumor recurrence was eight times higher in patients with GST-pi–positive breast tumors, as the relative risk was 8.1. Conversely, Bcl-2 expression reduced the risk of recurrence in breast cancer patients (relative risk of .31).

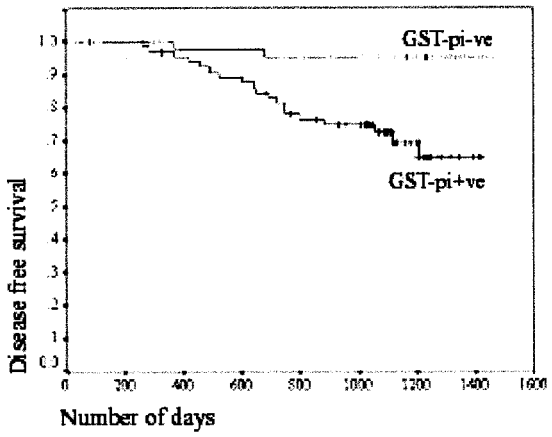

FIGURE 2. Disease-free survival in patients with GST-pi–positive tumors was worse than that of patients with GST-pi–negative tumors ($P = .002$).

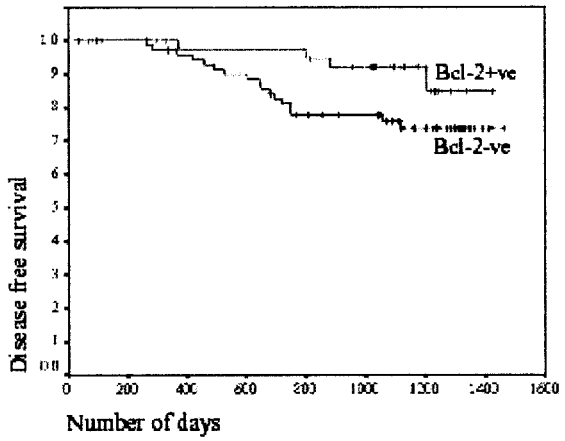

FIGURE 3. Disease-free survival in patients was not affected by Bcl-2 status ($P = .08$).

TABLE 3. Treatment Regime of Breast Cancer Patients after Surgery as Stratified By Bcl-2 and GST-pi Status

Biomarker Status	No Chemotherapy	With Adjuvant Chemotherapy
GST-pi positive	27	40 (22 on CMF)
GST-pi negative	18	31 (14 on CMF)
Bcl-2 positive	19	24 (12 on CMF)
Bcl-2 negative	26	47 (24 on CMF)

CMF = cyclophosphamide/methotrexate/5-fluorouracil regime.

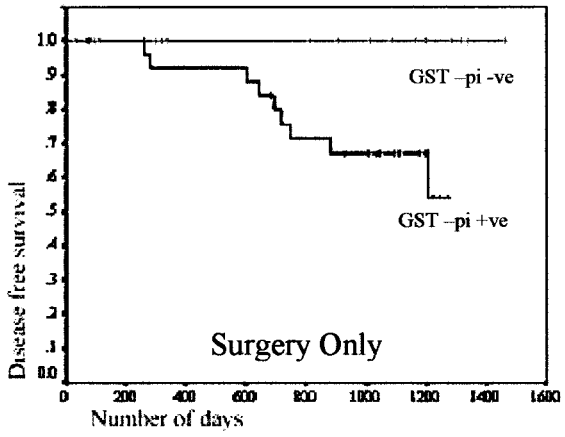

FIGURE 4. Disease-free survival in patients with GST-pi–positive tumors was inferior to that in patients with GST-pi–negative tumors who underwent surgery without chemotherapy ($P = .02$).

DISCUSSION

In the present study, GST-pi overexpression was detected in 58% of the breast cancer tissues examined. This finding is similar to that reported in Caucasian populations (17, 18). We observed that GST-pi expression was not associated with estrogen receptor status with a larger sample, although in a previous smaller study, we found that GST-pi expression was associated with estrogen receptor negativity (13). This may be due to subtle differences in tumor characteristics between the two samples, as differences in patient selection could result in the presence or absence of this association.

Only 37% of the breast cancers exhibited Bcl-2 overexpression, compared with 49% (19) and 63% (20) in two studies that used the same cutoff to define Bcl-2 overexpression. Whether such varia-

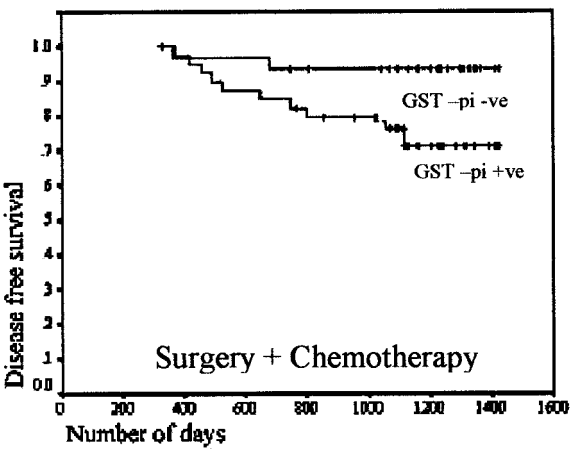

FIGURE 5. Disease-free survival in patients with GST-pi–positive tumors was worse than that of patients with GST-pi–negative tumors in the group who had adjuvant chemotherapy ($P = .04$).

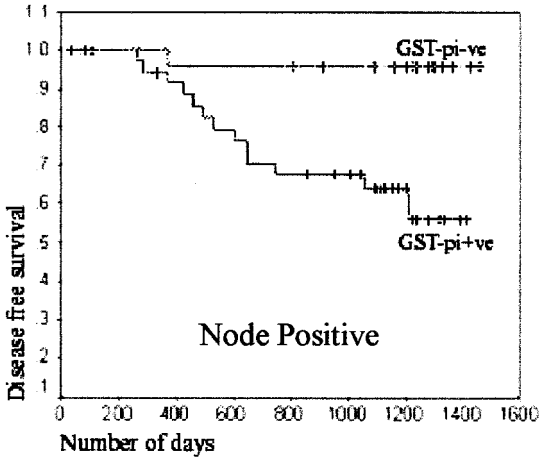

FIGURE 6. Disease-free survival in node-positive patients was significantly correlated with GST-pi immunoreactivity ($P = .002$).

TABLE 4. Relation of GST-pi Expression with Clinico-Pathological Factors in Axillary Lymph Node Positive Cases

Clinico-Pathological Factors	Number of Patients	GST pi-Positive	GST pi-Negative	*P* Value
Primary tumor				.652
T1	9	5	4	
T2	42	17	25	
T3 and T4	12	5	7	
Estrogen receptor				.434
Positive	39	18	21	
Negative	23	8	15	
Histologic grade				.285
I	8	2	6	
II	24	13	11	
III	31	12	19	

TABLE 5. Cox's Multivariate Analysis of Disease Free Survival in Breast Cancer Patients

Clinico-Pathological Factors	*P* Value
Lymph node status	.307
Estrogen receptor status	.374
Bcl-2 status	.067
GST-pi status	.006
Tumor stage	.971

tions were due to population differences await further confirmatory studies. Nevertheless, consistent with those studies, a higher proportion of Bcl-2–overexpressing tumors remains associated with estrogen receptor–positive tumors and tumors with better prognostic characteristics such as smaller size.

GST-pi immunoreactivity was observed to be diffusely located in the cytoplasm in most of the Bcl-2–negative tumors. In contrast, there appeared to be an increased nuclear localization of the GST-pi protein in Bcl-2–overexpressing tumors, as clearly demonstrated by two-color immunofluorescence with confocal microscopy. One plausible explanation for this observed phenomenon is that the Bcl-2 protein has been implicated as a regulator of transport through the nuclear pore (21). GST-pi, which has a molecular weight of 22 kDa, has the potential to gain entry into the nucleus through the nuclear pore complex (22). The precise role of GST-pi in the nucleus has not been ascertained, although an increased amount of nuclear GST-pi expression has been observed in breast cancer cells resistant to doxorubicin and *cis*-diamminedichloroplatinum (II; 23). The findings of that study showed that GST-pi was transferred to the nucleus when cells were exposed to the anticancer drugs, thus preventing DNA damage by inhibiting DNA intercalation and DNA cross-linking.

Apoptosis is a fundamental biological process that plays an important role in carcinogenesis (24). It is well established that this process could be triggered by a variety of agents, including oxidants (25). Oxidative damage to cells is known to be blocked by antioxidants, which have the capacity to scavenge reactive oxygen species (26). GST-pi is an antioxidant that is known to inhibit c-Jun *N*-terminal kinases that are required for maximal induction of apoptosis by DNA damaging agents, a pathway which is mediated by Bcl-2 (27). In addition, the GST family of enzymes has the capacity to prevent oxidative damage by catalyzing conjugation of electrophiles (28). However, we did not find a significant correlation of GST-pi expression with apoptosis when evaluated by multivariate analysis.

On the other hand, a higher Bcl-2 immunoreactivity was associated with a lower extent of apoptosis, a finding which is similar to that reported by Vakkala *et al.* (29). Suppression of apoptosis is reported to increase mutation frequency (30), and loss of apoptosis is associated with accumulation of oncogenes (31). By delaying cell death, the Bcl-2 protein may help to promote the accumulation of mutations, allowing cancer cells to acquire a more malignant phenotype. In view of its anti-apoptotic effects, Bcl-2 expression would apparently promote tumorigenesis. However, Bcl-2 is generally accepted as a favorable prognostic marker in breast carcinoma (32). In our study, Bcl-2-positive cases appeared to have improved disease-free survival (though not statistically significant). This is understandable, as prognosis is influenced by a host of other factors besides apoptosis. Although dysregulation of apoptosis is known to play an important role in metastasis (33), yet we observed no correlation of lymph node metastasis with Bcl-2.

It has also been demonstrated that the more aggressive infiltrative ductal carcinoma has a lower degree of apoptosis and higher proliferative activity compared with the case of intraductal carcinoma (34). This contrasts with our finding, in which a higher histological grade was observed to be associated with a raised apoptotic index. In his review, Lipponen (35) stated that increased apoptotic values in breast cancer are related to high histological grade and that the apoptotic index shows a positive correlation with indicators of cell proliferation such as mitotic index. Mitotic activity is one of the factors used in determining histological grade (12), and this could explain why histological Grade III tumors had higher apoptotic values than Grade I and II breast tumors.

The GST family of enzymes has long been implicated in chemotherapeutic drug resistance (36). Chemoresistance is a major cause for failure in cancer therapy, and modulation of cellular proteins such as GST-pi involved in detoxification has been suggested as one of the mechanisms that contribute to drug resistance (37). GST-pi expression has been reported to be related to clinical drug resistance (38). In our study, we found a significantly poorer disease-free survival in patients with GST-pi–positive breast tumors who received adjuvant chemotherapy after surgery, as compared with patients who had GST-pi–negative tumors.

Earlier on, Gilbert *et al.* (5) suggested that increased GST-pi expression could be an important predictor of early recurrence and death in node-negative breast cancer patients. We observed that patients with GST-pi immunopositivity had a higher relative risk of recurrence (8 times) when compared with their GST-pi–negative counterparts. This finding is in accord with that reported by Silverstrini and co-workers (18), who found that the risk of local recurrence at 6 years was higher for patients with tumors exhibiting elevated levels of GST-pi protein. On the other hand, our finding of a

reduced risk of recurrence in breast cancer patients with Bcl-2 expression (although the *P* value was marginally significant) is in concert with that reported by Castiglione and colleagues (39). They observed that a positive Bcl-2 status had a favorable impact on recurrence-free survival in European patients with breast cancer.

In conclusion, GST-pi expression appears to be an independent predictor of poor prognosis in breast cancer patients. Disease-free survival was worse in patients with GST-pi–positive breast tumors, and the relative risk of tumor recurrence was higher. GST-pi was linked with unfavorable disease outcome in node-positive breast cancer patients. It would appear that GST-pi can be added to the list of new and promising prognostic factors, such as tumor angiogenesis, epidermal growth factor receptor, and transforming growth factor alpha, that provide significant information in the clinical management of breast cancer patients (40).

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MINIREVIEW

Clinicopathological Significance of Metallothioneins in Breast Cancer

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Metallothioneins (MTs) are a family of metal binding proteins that play an important role in maintaining transition metal ion homoeostasis, redox balance in the cell and fundamental cellular processes such as proliferation and apoptosis. In humans, there are 4 groups of MT proteins which are encoded by 10 functional MT isoforms. In breast tissues, MT is primarily expressed in myoepithelial and malignant epithelial cells. Immunohistochemical studies have revealed that 26% to 100% of invasive ductal breast cancers express the MT protein. The MT-1F and MT-2A isoforms have been reported to be associated with higher histological grade in breast cancer, whereas higher MT-1E mRNA expression was found in estrogen receptor-negative tumors compared to their estrogen receptor-positive counterparts. A number of studies have shown that MT expression in breast cancer is associated with poorer prognosis. In addition, metallothionein expression may have a potential role in protecting the breast cancer cell from chemotherapeutic threats to survival. (Pathology Oncology Research Vol 10, No 2, 74–79)

Keywords: MT isoforms, biochemistry, biomarker, prognosis, chemoresistance, carcinogenesis

Introduction

Metallothioneins (MTs) are low molecular weight proteins of 6 to 7 kDa, with about 30% consisting of cysteine residues and no aromatic amino acids. The nomenclature for MT proposed by Kagi et al, define MTs as "polypeptides resembling equine renal metallothionein in several of their features".[1] They contain conserved sequences of cysteine residues juxtaposed with basic amino acids, such as lysine and arginine,[2] and these form metal-binding tetrahedral thiolate structures with special affinity for transition metals.[3]

The classification of MTs into families, subfamilies, subgroups and isoforms are based on sequence similarities and phylogenetic relationships.[4] In humans, MTs are encoded by a family of genes consisting of 10 functional MT isoforms which are located on chromosome 11q13.[5] The encoded proteins are classified into four groups, MT-1, MT-2, MT-3 and MT-4 proteins.[6,7] The functional genes of MT-1 encode MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H and MT-1X isoforms, whilst only one of the MT-2 genes, MT-2A, is functional. MT-3 is preferentially expressed in neural tissues[8] and MT-4 expression appears to be limited to squamous epithelial cells.[9]

Biochemical properties of MT

All MTs have characteristic cys-x-cys, cys-x-y-cys, and cys-cys sequences, where x and y represent non-cysteine amino acids. Mammalian MTs are believed to bind a total of seven bivalent metal ions through thiolate coordination in two separate clusters.[10] To date, complete three-dimensional structures which have been elucidated for rabbit and rat MT-2,[11-13] confirmed the presence of two separate clusters, *viz*, beta-domain comprising amino acid residues 1 to 30 and three metal ions, and alpha-domain containing amino acid residues 31 to 61 and four metal ions. Because of its metal binding properties, metallothionein has been postulated to be involved in cellular homoeostatic control and regulation of trace elements. In mammals, zinc-metallothionein complexes appear to be the predominant form.[14] However, the ways in which zinc distribution in

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the cell is regulated and the mechanisms of zinc transfer from protein to protein are currently not well known.[15] It has been shown that zinc is easily displaced by other metals ions, such as lead and cadmium, by virtue of its low binding affinity with the apoenzyme.[16] Yet, the binding affinity *in vivo* appears to vary depending on the nature of stress experienced by the cell,[17] suggesting that transition metal ion homoeostasis is actively modulated, rather than a passive chemical process. It is also believed that the cysteine sulfur forming ligands to zinc can be reduced or oxidized with concomitant binding or release of zinc, respectively, and such oxidoreductive mechanisms may link metallothionein function with specific cellular signals.[18]

Metallothioneins can also serve as a redox buffer. The metal binding thiolate clusters have a low redox potential and are readily oxidized by cellular oxidants.[19] It has been shown that MTs can scavenge superoxide and hydroxyl radicals in a manner similar to thiol containing molecules, such as N-acetylcysteine and glutathione.[20,21] The binding of transition metals displaying Fenton reactivity (Fe and Cu) can also reduce oxidative stress. As a result, MT overexpression confers protection against free radical induced DNA damage,[22] and lipid peroxidation.[23,24]

The specific functional roles of each of the MT isoforms are not precisely known. Whereas mRNA of MT-1A, MT-1E, MT-1X, and MT-2A genes are expressed in normal prostate,[25] MT-1F, MT-1G and MT-1H mRNAs are additionally expressed in breast myoepithelial cells.[26] Interestingly, although metal response elements are present in the promoters of all MT genes,[27] not all MT genes are responsive to metal induction. MT-1A and MT-1E isoforms are up-regulated after exposure to cadmium and zinc,[28] whereas MT-1A and MT-1X are induced by arsenic[29] in the same cell line, and a different MT expression pattern is seen when different cell lines experience similar heavy metal exposure.[30] MT-1E, MT-1X and MT-2A isoforms were increased in PMC42 breast cancer cells that were resistant to copper and zinc toxicity.[31] Recently, a significant variant MT-1H isoform with amino acid replacements and notable changes in the secondary protein structure was reported in breast cancer cells.[26]

MT expression in human breast cancer

MT expression is routinely visualized immunohistochemically using antibodies raised against the E9 epitope, which is conserved in both MT-1 and MT-2 isoforms. In a normal breast lobule that typically comprises bilayered ductules/acini (an inner epithelial layer and outer myoepithelial cells), strong nuclear and cytoplasmic MT immunopositivity was observed in myoepithelial cells and only rarely, in epithelial cells lining the large ducts.[32,33] Similarly, in other benign breast lesions such as adenosis, sclerosing adenosis and papilloma, only myoepithelial cells were shown to express MT.[34] Lobular cancer cells from in-situ or invasive tumors, showed weak to no expression of MT as well.[34,35] In contrast, a significant proportion of ductal breast cancers exhibited MT immunopositivity. Studies revealed that 26% to 100% of invasive ductal breast cancers express MT.[36-39] If a component of ductal carcinoma-in-situ was found in tumor tissues, the retained myoepithelial cells around these in situ islands were strongly highlighted immunohistochemically. In addition, MT expression was present in the in-situ cancer cells, with similar staining distribution and intensity to the surrounding invasive elements.[33]

The expression of different isoforms of MT mRNA in the breast cancer cell cytoplasm could also be demonstrated by in-situ hybridization on paraffin sections. Using RT-PCR on MT-expressing breast cancer tissues, the average quantity of MT-2A mRNA was found to be highest amongst the MT-1 and MT-2 isoforms and MT-1B mRNA was not detectable in all the samples.[38,40] Interestingly, MT-3 was also found to be expressed in 73% of breast cancers,[41] although it is not expressed in normal breast tissue.

Role of MT in breast carcinogenesis

The potential role of MT in carcinogenesis has been well appraised by Cherian and co-workers, who were also the first research group to establish MT expression in human tumors.[42-45] As MT is known to influence tumor growth by affecting both cell proliferation and death, which are fundamental processes in carcinogenesis,[46,47] its role in tumors has attracted a lot of attention in recent years.

MT expression in many tissues of fetal mammals is higher than that seen in adults.[48] In human colonic cancer cells, it has been demonstrated that metallothionein expression is increased 2-3 fold in proliferating cell compartments compared to growth inhibited cells, and peak expression occurs during late G1 and G1/S transition phases.[49] The level of combined MT-1 and MT-2 expression in breast cancer tissue, and more specifically, the MT-2A isoform, correlate with increased proliferation indicated by Ki-67 immunopositivity.[38] It was demonstrated that overexpression of MT-2A in breast MCF-7 cells resulted in a 2-fold increase in cell multiplication,[50] whilst over-expression of MT-1E and MT-3 in breast cancer cell lines did not affect proliferative rate. In fact, in two cell lines studied, MT-3 over-expression resulted in growth inhibition.[51]

MT expression has been linked to reduced apoptosis in hepatocellular carcinoma[52] and nasopharyngeal carcinoma.[53] Although the relationship is not seen in breast cancer tissues,[38] interestingly, anti-sense down-regulation of MT-2A in MCF-7 cells was associated with both reduced cell growth and increased apoptosis with lower bcl-2 protein levels and decreased expression of c-myc mRNA tran-

Table 1. **MT as a prognostic marker in breast cancer**

Reference	*Country*	*Prognosis in relation to to high MT expression*
Ioachim et al., 2003[39]	Greece	Limited prognostic value
Vazquez-Ramirez et al., 2000[37]	Spain	Poor prognosis
Zhang et al., 2000[36]	China	Poor prognosis and higher histological grade
Oyama et al., 1996[64]	Japan	No correlation with prognosis
Goulding et al., 1995[66]	UK	Poor prognosis
Haerslev et al., 1995[63]	Denmark	Poor prognosis, axillary lymph node involvement, negative progesterone receptor status and higher histological grade
Fresno et al., 1993[32]	Spain	Poor prognosis, negative estrogen receptor status and higher histological grade

scripts compared to controls.[51] It is possible that whilst MT expression may influence both proliferation and apoptosis, there are other more important factors that are called into play when apoptosis is triggered in breast cancer.[54]

The mechanism by which MT exerts its effects is not precisely known. MT was found to interact specifically with the p50 subunit of NF-κB in MCF-7 cells,[55] and to inhibit the binding of NF-κB to DNA following TNF activation.[56] The effect appears to be mediated by both MT-1 and MT-2 isoforms.[57,58] The possibility that MT might be able to interact with other proteins involved in cell proliferation and apoptosis was raised when MT-2A was also found to interact with esophageal cancer related gene 2 (ECRG2).[59]

There also appears to be a functional link between MT and the p53 tumor suppressor gene.[60] In the presence of zinc, MT facilitates normal functional p53 activity by zinc transfer between MT and p53, resulting in the maintenance of a DNA-binding conformation.[61] However, the transfer may be in the reverse direction under conditions of zinc depletion,[62] resulting in the disruption of the conformation of the DNA-binding domain and a phenotype similar to many mutant forms of p53. It has also been suggested that p53 and oestrogen-receptor may play a part in the expression and induction of metallothionein in human epithelial breast cancer cells.

Association of MT with pathological parameters and molecular markers of breast cancer

MT expression in breast cancers has been studied in association with common clinico-pathological parameters used in breast cancer prognosis and other common oncogenes. High overall MT expression was consistently associated with increased tumor grade and more severe nuclear pleomorphism compared to the low MT expressing counterparts.[32,36,38,63,64] Some studies have also shown an inverse correlation between MT expression with estrogen receptor[32,64] and progesterone receptor content.[63,65] On the other hand, most studies showed no statistically significant association of MT expression with tumor size and with presence of lymph node metastasis at diagnosis,[38,39,64,66] although there is a numerical tendency for breast tumors of poorer stage to be more highly MT expressing.[38,39]

In breast cancer tissues, MT expression has also been studied in relation to the expression of tumor suppressor proteins (p53, pRb, Bcl-2), extracellular matrix components (type IV collagen, laminin), invasion- and tissue modeling-related genes (fibronectin, cathepsin D, CD44, matrix metalloproteinase-3), as well as growth factor receptors (c-erbB2, EGFR).[37,39,65,67] However, none of these biomarkers were associated with MT expression.

Looking into specific MT isoforms, Bay et al found that increased MT-1F and MT-2A mRNA were separately associated with higher histological grade, but not with patient age and lymph node status.[38,40] Higher MT-1E mRNA expression was found in estrogen receptor negative tumors compared to estrogen receptor positive ones.[68] However, there was no significant difference in MT-1E expression between progesterone receptor positive and progesterone receptor negative tumors.

MT as a marker of prognostication in breast cancer

Higher MT expression in breast cancers has generally been shown to predict worse survival for patients (*Table 1*). Fresno et al.[32] found that patients with MT expressing breast cancers had decreased overall survival and shorter disease-free survival if the cancers were also estrogen receptor negative or lymph node negative. Other studies that included 72 to 478 patients,[36,37,39,63,66] have found worse prognosis associated with MT expression with the entire study population included in the analyses. A single study consisting of 92 patients found no statistically significant different in survival when the patients were stratified according to MT expression levels by univariate analysis.[64]

Multivariate analysis, including other clinico-pathological parameters, were reported only in a few studies,[37,63] and these showed that MT expression did not provide addi-

tional prognostic information with all other factors considered. This was probably due to the strong association of MT expression with other factors predicting poor prognosis (such as tumor grade) in the studies.

MT and chemoresistance

Metallothionein has been extensively studied as a possible mediator of chemotherapy resistance.[69] In solid tumors treated uniformly with cisplatin-based chemotherapy, such as esophageal squamous cell carcinoma,[70] urothelial transitional cell carcinoma,[71] and small cell lung cancer,[72] metallothionein expression in the tumors have been associated with improved survival. It was felt that chemotherapy resistance to cisplatin is mediated, in part, by transfer of platinum from cisplatin to metallothionein, resulting in inactivation.[73] However, when ovarian cancer patients were treated with several chemotherapy regimes (some cisplatin-based), such a protective effect was not observed.[74,75] This suggests that the chemoprotective effect of metallothionein is probably regime specific.

Recent evidence suggests that metallothionein also reduces etoposide-induced apoptosis in lung and liver cancer cell lines, and the effect was increased with higher MT levels induced by pre-treatment with zinc or cadmium.[76] The mechanism by which metallothionein defer cell death from etoposide exposure is still not fully elucidated. However, little is known about the effect of metallothionein expression on the sensitivity of breast cancer cells to common chemotherapeutic agents used in the treatment of breast cancer. As drug resistance is a multifactorial phenomenon, the provision of direct and compelling evidence on the role of MT in chemoresistance in tumors is a difficult task.[77]

Conclusion

Much remains to be learnt about the function of metallothionein in breast carcinogenesis and chemotherapy resistance, especially with regard to what role each of the isoforms performs in these processes. This may help in the development of a specific therapeutic agent that aims to correct the abnormal expression of metallothionein in breast cancers. Selective up-regulation of metallothionein in non-cancer tissues can also be explored further, so that existing treatment options may be utilized to greater effect.

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GST-pi expression correlates with oxidative stress and apoptosis in breast cancer

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Abstract. Glutathione S-transferase (GST) is known to play a key role in the detoxification and reduction of reactive oxygen species (ROS). Thus, we assessed GST activity and GST-pi expression in relation to oxidative stress and apoptosis in breast cancer. Tumor tissues from 32 breast cancer patients were evaluated for GST activity and thiobarbituric acid reactive substances (TBARS) that are by-products of oxidative stress. Four-micron sections of formalin-fixed, paraffin embedded tumors were stained immunohistochemically with anti-GST-pi. Apoptotic cells were detected by *in situ* end labeling of DNA fragments using a commercial kit. TBARS levels were significantly higher in breast cancers of older patients. GST-pi expression was up-regulated in breast cancers that exhibited higher oxidative stress and associated with higher GST activity. Apoptosis in GST-pi negative tumors was not correlated with GST activity, but GST-pi positive tumors within the same range of oxidative stress showed a reduction in apoptosis as well as an increased GST activity. This correlation was absent in GST-pi positive tumors experiencing higher oxidative stress. GST-pi expression may influence the level of GST activity and delay apoptosis in breast cancer. However, GST-pi expression in tumors with higher levels of oxidative stress may not be sufficient to abrogate the deleterious effects of ROS.

Introduction

Oxidative stress arises when the production of reactive oxygen species (ROS) exceeds the scavenging capacity of cellular enzymatic and non-enzymatic anti-oxidant defense. Accumulation of ROS causes lipid peroxidation, protein modification and genetic mutations (1). Higher levels of oxidative stress may trigger apoptosis via the mitochondrial pathway (2). In this death receptor-independent pathway, ROS induces the release of cytochrome C and changes in mitochondrial membrane permeabilization. Release of cytochrome C initiates a cascade of enzymatic events resulting in the activation of caspase 3 and culminating in apoptosis. Modulation of anti-oxidant defense against ROS appears to be important in cancer cells. For instance, inhibition of superoxide dismutase in human leukemia cells by certain estrogen derivatives has resulted in reduced cell survival (3).

The glutathione S-transferases (GST) are a super-family of enzymes, with 8 distinct gene families (namely, alpha, mu, theta, pi, sigma, zeta, kappa and chi) encoding the cytosolic form of the enzyme found in human beings. Among its activities, these enzymes confer anti-oxidant protection through the neutralization of the toxic carbonyl-, peroxide- and epoxide-containing metabolites produced within the cell by oxidative stress via conjugation with glutathione (4). They are also responsible for a substantial proportion of total glutathione peroxidase activity in human tissues (5). In particular, glutathione S-transferase P1-1 (GST-pi) is associated with altered and variable expression in liver, renal, prostate and breast cancers (6) and linked with nasopharyngeal and breast cancers of a more aggressive phenotype (7-9).

Apoptosis has been investigated as a possible pathway that could be manipulated for the treatment of breast cancer (10). However, modulation of apoptosis by varying levels of anti-oxidant enzyme expression in breast cancer has not been extensively documented. In this study, we investigated the association between GST activity and GST-pi expression in relation to oxidative stress and apoptosis in breast cancer.

Materials and methods

Patients. Thirty-two breast cancer samples were obtained from patients who underwent mastectomy without neo-adjuvant treatment at the Singapore General Hospital. Their ages ranged from 44-85 years, with a median of 55.5 years. All the tumors were classified histopathologically as invasive breast ductal cancers. Table I summarizes the clinico-pathological characteristics of the cases. Immediately after surgery and gross pathological examination, sections of at least 1 cm^3 were rapidly frozen in liquid nitrogen and stored until further use for the measurement of total GST activity and quantitation of thiobarbituric acid reactive substances (TBARS) for each of

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Table I. Clinicopathologic characteristics of 32 breast cancer tissues in relation to GST activity, TBARS level and apoptotic index (p-values).

Clinico-pathological characteristics	N	GST activity	TBARS	Apoptotic index
Age				
≤50 years	5			
>50 years	27	0.617	0.006	0.305
Lymph node metastasis				
Absent	12			
Present	20	0.460	0.654	0.626
Hormone receptor status				
Absent	11			
Present	21	0.370	0.184	0.905
Grade				
I and II	17			
III	15	0.748	0.113	0.806
Size of tumour				
≤2cm	7			
>2cm	25	0.327	0.802	0.236

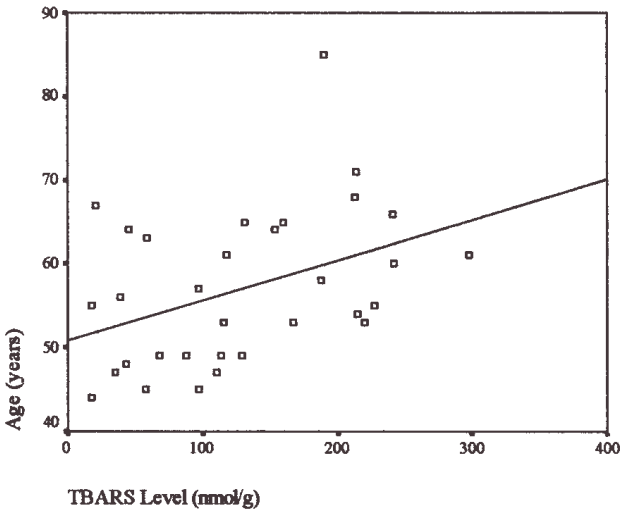

Figure 1. Positive correlation between age of patients and TBARS levels in cancer tissues (rho=0.407, p=0.021). Higher TBARS levels were found in breast cancer tissues from older women.

the tumors. The remaining tissues were fixed in formalin and embedded in paraffin for histological examination.

Tissue homogenate. Frozen breast cancer tissues were thawed on ice, blotted with filter paper and weighed. They were then homogenized in sufficient 50 mM phosphate buffer at pH 7.4, under standard conditions to make a 10% homogenate. The homogenate was centrifuged at 40,000 rpm at 0˚C, to obtain a cell-free supernatant.

GST activity assay. Total GST activity was determined by measuring the rate of conjugation of glutathione (GSH) and 1,2-chloro-2,4-dinitrobenzene (CDNB). Cell-free tissue homogenate (10 µl) was added to a mixture of 950 µl of 0.1 M phosphate buffer pH 6.5, 20 µl of 50 mM CDNB in ethanol and 20 µl of 50 mM GSH in phosphate buffer. The reaction at ambient temperature of 25˚C was monitored by the rise in optical density at 340 nm. Correction for non-catalyzed reaction was made by subtracting the rate of change of optical density without enzyme from that with tissue homogenate. One unit of GST activity is defined as the amount of enzyme necessary to conjugate 1 nmol of CDNB with 1 nmol of GSH per min.

TBARS analysis. Quantifying thiobarbituric acid reactive substances (TBARS) from tissue extract is a standard assay for lipid peroxidation. Breakdown products of lipid peroxidation react with 2-thiobarbituric acid to form an easily detectable chromogen. Briefly, a reaction mixture of total volume 3 ml was constituted from 0.2 ml of cell-free tissue homogenate, 0.2 ml of 8.1% sodium dodecylsulfate, 1.5 ml of 1% phosphoric acid, 0.1 ml of distilled water and 1 ml of 0.6% thiobarbituric acid, was heated for 45 min at 100˚C, and 4.0 ml of n-butanol was then added to extract the pink chromogen obtained at room temperature (RT). The fraction dissolved in n-butanol was separated from the rest of the reaction mixture by centrifugation at 1000 g for 5 min. The optical density of the n-butanol layer was determined at 535 nm.

GST-pi immunohistochemistry. Paraffin-embedded sections were stained immunohistochemically for GST-pi using the polyclonal antibody anti-GST-pi antibody (Dako, USA) at 1:200 dilution. After washing and incubation with the appropriate secondary antibody, avidin-biotin-peroxidase complex was applied for 1 h at RT to amplify the specific binding of primary antibody. Visualization was achieved by incubating with 3,3' diaminobenzidine tetrachloride (Sigma) as the peroxidase substrate. The sections were then counter-stained with methyl green. GST-pi expression was considered to be positive when >10% of tumor cells exhibited cytoplasmic or nuclear staining.

In situ detection of apoptosis. For the detection of apoptosis in tissue sections, the commercially available TdT-FragEL™ DNA Fragmentation Detection kit (Oncogene Research Products, USA) was used. Briefly, tissue sections were incubated with 20 µg/ml proteinase K for 20 min at RT, followed by quenching of endogenous peroxidase with 3% H_2O_2. Subsequently, sections were incubated with TdT enzyme containing biotin labeled and unlabeled at 37˚C for 90 min. The rest of procedure was carried out as in the manufacturer's instructions. The apoptotic index was defined as the number of apoptotic nuclei per 100 cancer cell nuclei.

Statistical analysis. The Mann-Whitney test was used to compare the mean GST activity, TBARS level and apoptotic index for different groups of breast tumors. Correlation between

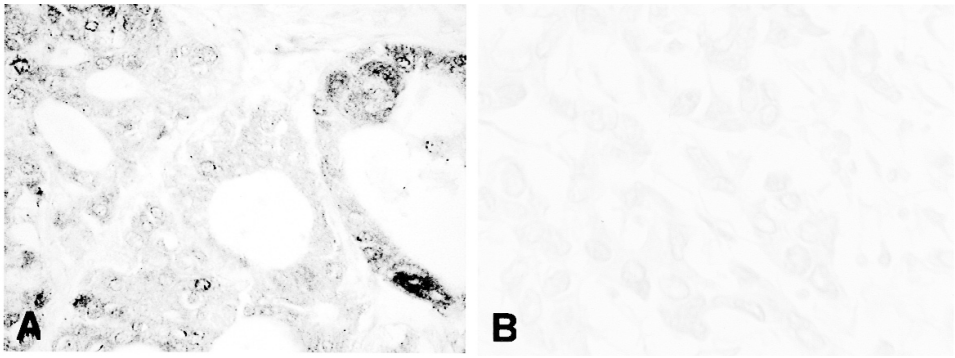

Figure 2. Detection of GST-pi expression in breast cancer by immunohistochemical staining with GST-pi antibody. Methyl green counterstain. A, Positive GST-pi immunostaining, original magnification x180; B, negative GST-pi immunostaining, original magnification x240.

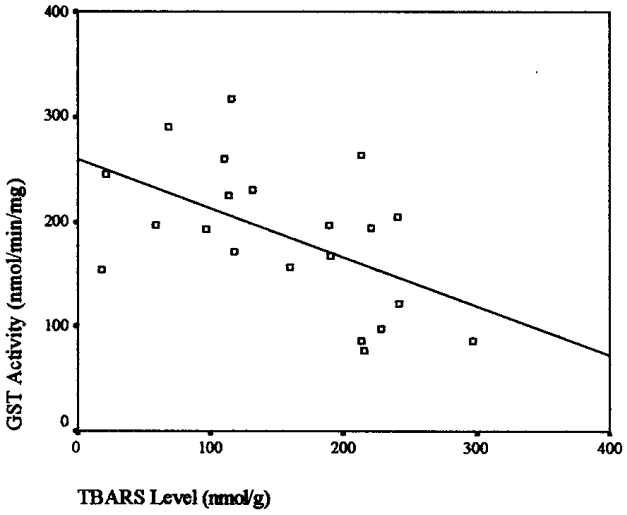

Figure 3. Inverse correlation between GST activity and TBARS level in GST-pi positive breast cancers (rho= -0.535, p=0.012). Higher TBARS levels in GST-pi positive tumors was associated with lower GST activity.

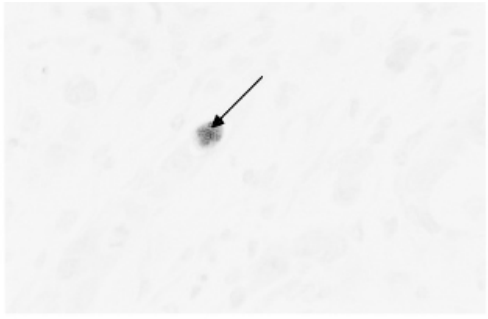

Figure 4. Detection of apoptosis by *in situ* end labeling of DNA fragments in breast cancer tissues. Positive cells are stained brown by diaminobenzidine, a chromogen substrate which reacts with the labeled cells at the site of DNA fragmentation. An apoptotic cell (arrow) is observed in this tissue section. Original magnification x240.

two continuous variables were investigated using the Pearson's test for bivariate correlations. The software used was SPSS software for Windows version 11.0. $p<0.05$ was considered to be statistically significant.

Results

There was marked variation in the GST activity among the 32 breast cancers analyzed. GST activity ranged from 76-317 nmol/min/mg protein with a mean value of 170 nmol/min/mg protein. There were no significant differences in GST activity between subgroups of cancers based on age at diagnosis, lymph node status, hormone receptor status, size and grade of tumor (Table I).

The mean TBARS level was 129 nmol/g wet weight with a range of 18-298 nmol/g wet weight. Interestingly, TBARS levels were higher in breast cancers of older patients (rho=0.407, p=0.021; Fig. 1). However, there were also no significant differences in the level of TBARS between subgroups of breast cancer stratified according to common clinicopathological characteristics (Table I).

Positive GST-pi staining was observed in 21 tumors. Fig. 2A illustrates the typical immunostaining pattern in GST-pi positive breast cancer cells and Fig. 2B is representative of a GST-pi negative breast tumor section. GST activity in GST-pi positive tumors was higher than that of GST-pi negative tumors (187±15 nmol/min/mg vs. 137±17 nmol/min/mg, respectively, p=0.041). The mean level of TBARS was also higher in GST-pi positive tumors (155±17 nmol/g) as compared to GST-pi negative tumors (80±16 nmol/g, p=0.003). For GST-pi positive tumors, higher GST activity was linearly correlated with lower TBARS level (rho= -0.535, p=0.012; Fig. 3), but there was no significant correlation between GST activity and TBARS level in GST-pi negative tumors (p=0.06).

Apoptotic breast cancer cells detected by *in situ* end labeling of DNA fragments are shown in Fig. 4. Higher GST activity in breast cancer was associated with a decrease in apoptosis only in the subgroup of 11 GST-pi positive tumors with TBARS <175 nmol/g (rho= -0.607, p=0.048; Fig. 5). The cut-off level of TBARS was taken to be 175 nmol/g so that all GST-pi negative tumors could be categorized into one subgroup. Apoptotic rates in GST-pi positive tumors with higher levels of oxidative stress and GST-pi negative tumors varied independently of GST activity (p=0.840 and p=0.066; respectively).

Discussion

Oxidative stress experienced by a malignant cell is often greater than its benign counterpart. In addition to that produced

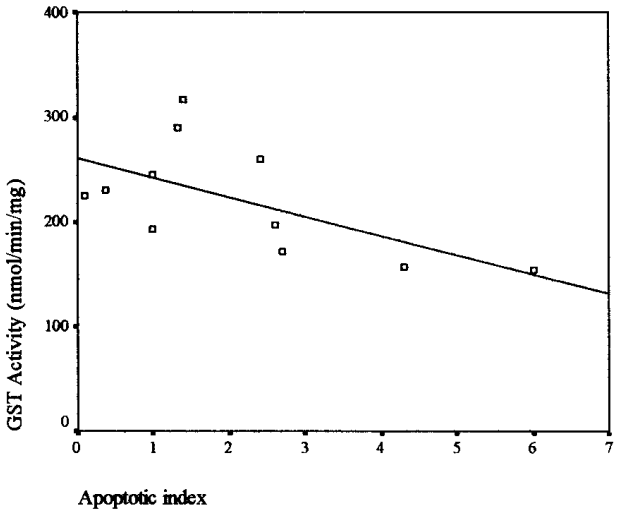

Figure 5. Inverse correlation between GST activity and apoptosis in GST-pi positive tumors with lower oxidative stress which is arbitrarily defined as TBARS <175 nmol/g (rho= -0.607, p=0.048).

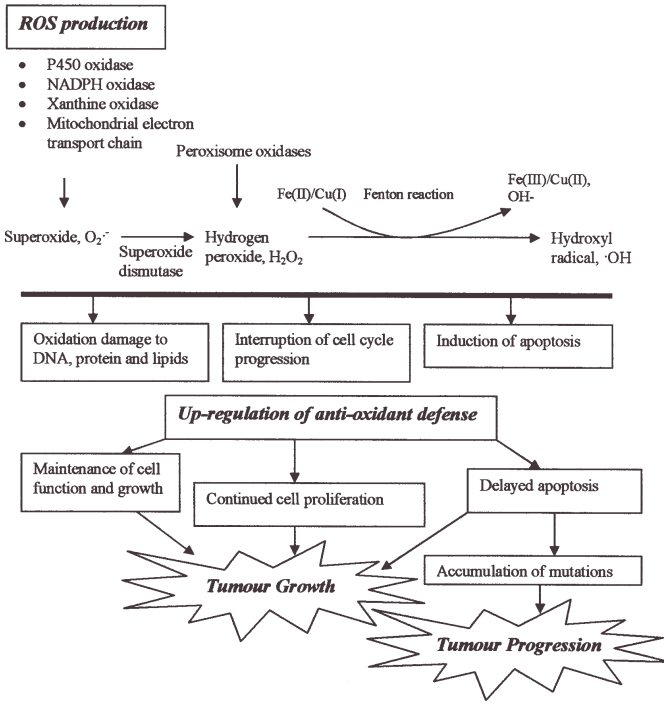

Figure 6. Effects of ROS and anti-oxidant defense against ROS.

during aerobic cellular metabolism, the malignant cell experiences additional pressures on cell survival because of genetic mutations, abnormal protein expression and extrinsic factors in the microenvironment. Activated leukocytes release ROS, such as nitric oxide which kills tumor cells (11). T-cells and macrophages release tumor necrosis factor (TNF) against tumor cells, inducing apoptosis, a process which may involve ROS. However, it is also known that TNF-induced caspase-3 activation in the TNF-receptor death pathway, is suppressed by transfection of cells with superoxide dismutase (12).

Indeed, markers of oxidative stress have been reported to be higher in breast cancers compared to their surrounding normal breast tissue (13). Substantial variation in oxidative stress levels in breast cancer, may be partly explained by increased exposure to oxidants in the systemic circulation. Assays of markers of oxidative stress in the sera of normal human subjects aged 20-70 years showed higher oxidative stress in older individuals (14). A similar trend was present in breast cancer tissues analyzed in our study, where tissues obtained from older patients had higher levels of TBARS. Although levels of oxidative stress were found to not be influenced by traditional pathological markers of tumor differentiation, including tumor grade and hormone receptor status, this would have to be confirmed with a larger sample size.

To protect against the deleterious effects of ROS (Fig. 6), the cell possesses a number of anti-oxidant defences, *viz*., biochemical anti-oxidants, metal ion binders and intra-cellular enzymes (including superoxide dismutase, glutathione peroxidase, catalase, thioredoxin reductase and GST). Mean activities of such enzymes have been found to be higher in breast cancer tissues compared to adjacent normal breast tissues (13) and benign breast tumors (15). In fact, the entire glutathione detoxification pathway was found to be up-regulated in untreated breast cancers compared to normal tissues and provided greater redox protection in the cancer cells as reflected in the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio (16).

Because of their broad specificity and key role in the detoxification and reduction of cytotoxic agents, GST has important constitutive function in tissues. Dysregulation and genetic polymorphisms in GST subfamilies are actively studied in many types of cancers (17,18). Amongst the members of the GST family, the expression of alpha class of GST in breast cancer is low, whilst the expression of GST-mu is consistent with the presence of germ-line polymorphism of the GST-mu gene. GST-pi expression, on the other hand, is widely variable in breast cancers (19,20). In the present study, tumors over-expressing GST-pi had higher total GST activity, suggesting that among the GST isoforms, GST-pi contributes significantly to variations in GST activity in breast cancer. The inverse correlation observed between TBARS and GST activity in GST-pi expressing tumors might also indicate a limited level of protection against (or reversal of) stress by GST-pi. Tumors with up-regulation of GST-pi expression appear to be those exposed to more oxidative stress (higher TBARS level).

However, the relationship between apoptosis and GST activity appears to be more complicated. The drive towards apoptosis is a complex process, involving other stimuli in addition to the balance between oxidative stress and anti-oxidant protection. It is also recognized that cellular response to extremes of oxidative stress may not be dose-dependent (21). Although we observed that apoptosis in GST-pi negative tumors was not correlated with GST activity, GST-pi positive tumors within the same range of oxidative stress showed a reduction in apoptosis with increased GST activity. This finding is consistent with that reported in the Jurkat T-cell line where partial inactivation of GST-pi was shown to favor apoptosis (22).

In conclusion, our findings suggest that GST-pi, an anti-oxidant enzyme, may have a role in delaying apoptosis in breast cancers. Interestingly, in GST-pi positive tumors experiencing higher oxidative stress, apoptotic rates varied independently of GST activity. Presumably, up-regulation of

GST activity in breast cancers experiencing higher levels of oxidative stress may not be sufficient to inhibit the deleterious effects of ROS.

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Y-box binding protein, YB-1, as a marker of tumor aggressiveness and response to adjuvant chemotherapy in breast cancer

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Abstract. The Y-box binding protein 1 (YB-1) regulates gene expression through transcription and translation. YB-1 has been shown to be associated with up-regulation of P-glycoprotein (Pgp), an ATP-binding transporter involved in multi-drug resistance. In this study, we determined the prognostic significance of YB-1 and its relationship with Pgp in patients with breast cancer. YB-1 and Pgp expression were evaluated by immunohistochemistry in resected specimens of infiltrative ductal breast cancers from 99 patients and 57 patients respectively and correlated with clinico-pathological parameters and adjuvant chemotherapy regimes. The antibody for the YB-1 protein was prepared by injecting a rabbit with a purified recombinant chicken YB1 protein. The relationship between YB-1 and Pgp was also evaluated by a computational approach using the Resonant Recognition Model (RRM). We found that breast tumors which were both estrogen receptor-negative and lymph node positive were associated with high YB-1 expression (P=0.017). In patients who did not receive adjuvant chemotherapy, recurrence risk was reduced in breast cancers having lower YB-1 expression (P=0.034), suggesting that high levels of YB-1 expression in breast cancer is associated with tumor aggressiveness. We were able to demonstrate a direct interaction between YB-1 and Pgp using the computer-based RRM. Interestingly, we found that patients who were on a chemotherapy regime which contained an anthracycline (a Pgp substrate) and subsequently developed recurrence, had a higher YB-1 score compared to patients on the Cyclophosphamide/Methotrexate/5-Fluorouracil regime (P=0.024). YB-1 expression in breast cancer may be a potential marker of chemoresistance and could possibly aid in selection of the appropriate adjuvant chemotherapy regime for breast cancers.

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Key words: breast carcinoma, immunohistochemistry, P-glycoprotein, prognosis, resonant recognition model, Y-box binding protein-1

Introduction

Y-box binding protein 1 (YB-1) has been stated as 'the most evolutionary conserved nucleic-acid-binding protein currently known' (1). YB-1 belongs to a group of DNA and RNA binding proteins that has a conserved cold-shock domain which interacts with inverted CCAAT boxes (Y-boxes) (1,2). As YB-1 regulates gene expression via transcription and translation (3), it is believed to play an important role in the cell cycle (4). YB-1 has also been linked to a number of cellular responses to stress and carcinogenic stimuli. It has been reported to have an affinity for depurinated and cisplatin modified DNA (5,6), as well as RNA damaged by reactive oxygen species (7). YB-1 has been found to translocate to the nucleus when the cell is exposed to UV irradiation (8) and heat (9). The protein is involved in redox-dependent transcription activation (10) and known to be up-regulated in cell-lines which are resistant to genotoxic agents (11,12).

The role of YB-1 in cancer progression has attracted attention in recent years. Increased YB-1 expression has been correlated with DNA topoisomerase IIα and proliferating cell nuclear antigen expression in human lung cancer (13) and colorectal cancer (14) and linked to markers of cellular proliferation in osteosarcoma (15). In addition, YB-1 is thought to promote metastasis by enhancing the transcription of gelatinase A, a matrix proteinase that facilitates cell migration (16). Moreover, expression of YB-1 protein has been reported to reflect chemosensitivity of ovarian serous adenocarcinoma (17). In this context, YB-1 expression has also been shown to be associated with Pgp expression in breast cancer cells, resulting in multi-drug resistance (18). Pgp is a member of the ATP-binding transporter family which is involved in the transport of potentially toxic xenobiotics (19).

Breast cancer is the second most common cause of cancer deaths in women around the world. Identifying aggressive

breast tumors with a high risk of distant metastasis is important for the institution of appropriate adjuvant treatment after surgery, thereby enhancing patient survival. Because of the association of YB-1 with biological processes which affect carcinogenesis and chemotherapeutic response, we have investigated the prognostic significance of YB-1 in human breast cancer tissues. The possible interaction between YB-1 and the CCAAT box in the promoter region of the multi-drug resistance gene MDR1 which encodes Pgp was also evaluated by computational tools.

Patients and methods

Patients and tumor samples. Resected specimens were obtained from 99 patients with infiltrative ductal breast carcinomas who had undergone mastectomy or lumpectomy without neoadjuvant treatment in the Singapore General Hospital between 1998 and 1999. The study was approved by the Institutional Ethics Committee. The age of the patients ranged from 33 to 86 years with a median age of 52 years. The patients had no distant metastasis at the time of diagnosis. Treatment decisions after surgery were based solely on established recommendations. Fifty-seven of the patients received adjuvant chemotherapy, of which the Cyclophosphamide/Methotrexate/5-Fluorouracil (CMF) regime was most commonly used (32 patients). The other patients received some form of anthracycline-based chemotherapy such as the Adriamycin/Cyclophosphamide regime (10 patients). Patient follow-up ranged from 33 to 1464 days with a median follow-up of 1112 days.

Cell culture. HeLa cells (American Type Culture Collection) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 5% fetal calf-serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

YB-1 antibody. Antiserum against YB-1 protein was prepared by injecting a rabbit with the purified recombinant chicken YB1 protein with N-terminal deletion (amino acids 50-326, Matsumoto *et al*, unpublished data). The IgG fraction of the antiserum was purified by protein G Sepharose column chromatography (Amersham Pharmacia).

Immunoblotting. HeLa cells were washed with phosphate-buffered saline and lysed by sonication in 5 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and 0.25 mM phenylmethylsulfonyl fluoride. Cell lysate (10 μg) was separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Nitrocellulose membranes were blocked for 2 h with 5% milk in TBST (10 mM Tris pH 8.0, 100 mM NaCl and 1% Tween-20) buffer. Membranes were then probed with a rabbit polyclonal antibody to recombinant chicken YB-1 protein (as described above) in 5% milk in TBST overnight at 4˚C. The membranes were washed with TBST and incubated with a goat anti-rabbit peroxidase conjugated antibody (Pierce, Rockford IL, USA) for 2 h at room temperature. Excess antibody was removed by further washes with TBST. The bound antibodies were visualized by chemiluminescense.

Immunohistochemistry. For detection of YB-1 expression, sections were pre-heated in 10 mM citrate-buffer at pH 6.0 before incubation with rabbit polyclonal antibody to recombinant chicken YB-1 protein. The antibody was used at a dilution of 1:200 and applied at room temperature overnight. Secondary antibody biotinylated anti-rabbit immunoglobulin (Dako, CA, USA; 1:200 dilution) was then applied for 30 min, followed by a 30-min incubation with biotin and avidin-peroxidase complex (Dako). The immunostaining was demonstrated using diaminobenzidine and hydrogen peroxide for 10 min. The sections were counterstained with hematoxylin. Pgp immunohistochemistry was performed for the 57 patients who underwent chemotherapy. It was detected using a mouse anti-human monoclonal JSB-1 antibody (Chemicon, CA, USA; 1:20 dilution) which reacts with a conserved cytoplasmic epitope of the protein. The immunohistochemical method used was optimized from that described by Faneyte *et al* (20). Dewaxed sections were microwave-heated for 25 min from cold. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 10 min and non-specific antibody binding blocked in 4% normal horse serum for 30 min. Primary antibody was then applied for 1 h. In the negative control, buffer solution (0.5 M Tris-buffered saline, pH 7.4) replaced the primary antibody. A normal liver section was used as positive control. Visualization was achieved by the usual method described above with hematoxylin as the counterstain.

Quantification of immunohistochemical staining. The stained slides were viewed using a x40 objective of a light microscope, and 10 random fields were selected and scored. Semi-quantitative determination of YB-1 expression was performed using an immunoreactive score modified from Janz *et al* (21). The immunoreactive score (range 0-15) is derived from the product of intensity of staining and percentage score. The intensity of staining was scored from 0 (no detectable immunoreactivity) to 3 (strong immunoreactivity). The percentage score which is the percentage of cancer cells expressing YB-1 was scored as such: i, <20%; ii, 21-40%; iii, 41-60%; iv, 61-80%; v, 81-100%. Pgp over-expression was classified as positive when cancer cells exhibited cytoplasmic or plasma membrane staining patterns (22).

Computational analysis. The Resonant Recognition Model (RRM) was used for computational analysis of direct interaction between YB-1 and the Y-box region of the MDR1 gene promoter. RRM is based on the theory that protein-protein or protein-DNA interaction depends on the resonant electromagnetic energy transfer at a specific frequency for each interaction (23). The sequences of seven YB proteins (24) were obtained from the National Centre for Biotechnology Information (NCBI) database (Table I). Three promoter sequences of MDR1 genes were retrieved from the Eukaryotic Promoter Database (EPD ID: EP35012, EP35017 and EP35016). The RRM power spectra were then calculated for each of the sequences. 'Consensus' cross-power spectra were calculated for the seven power spectra of the YB proteins, as well as for the three spectra of MDR-1 promoter sequences. The two spectra were then analyzed for any common frequency component.

Table I. Accession numbers of YB proteins (NCBI database).

YB-1 proteins	Accession no.
YB-1 human	P16991
DbpA human	P16989
YB-1 mouse	P27817
EF1 human	AAA30497.1
mRNP3 frog	P45441
mRNP4 frog	P21574
YB-1 frog	P21573

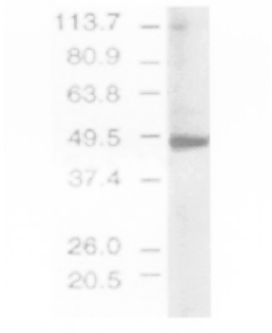

Figure 1. Western blot analysis of YB-1 protein in cell lysate from HeLa cells.

Statistical analysis. For statistical analysis, the SPSS software for Windows Release 11.0 was used. YB-1 scores were compared by non-parametric, Mann-Whitney U test. For the purpose of statistical analysis, the immunoreactive scores were classified into 3 groups: low, moderate and high levels of expression. The 25th percentile of the scores was taken as the lower cut-off, whilst the 60th percentile was used as the higher cut-off. As a result, immunoreactive scores 0-2 were considered low; immunoreactive scores 3-5, moderate; immunoreactive scores 6-15, high. For prognostication, the patients were divided into 3 discrete groups with lymph node status and estrogen receptor status as the parameters. The categorical variables were analyzed with the Fisher's exact test. Disease-free survival curves were plotted by the Kaplan-Meier method and the curves were examined by means of log-rank test.

Results

Specificity of YB-1 antibody for human YB-1 protein. The specificity of the anti-serum against purified recombinant chicken YB1 protein for human YB-1 protein in HeLa cells is shown in Fig. 1.

Expression of YB-1 and Pgp in breast cancer samples. YB-1 expression was detected in all 99 breast cancer samples, with 27 having low levels, 40 having moderate levels, and 32 having high levels of expression (Figs. 2A and B). Eleven

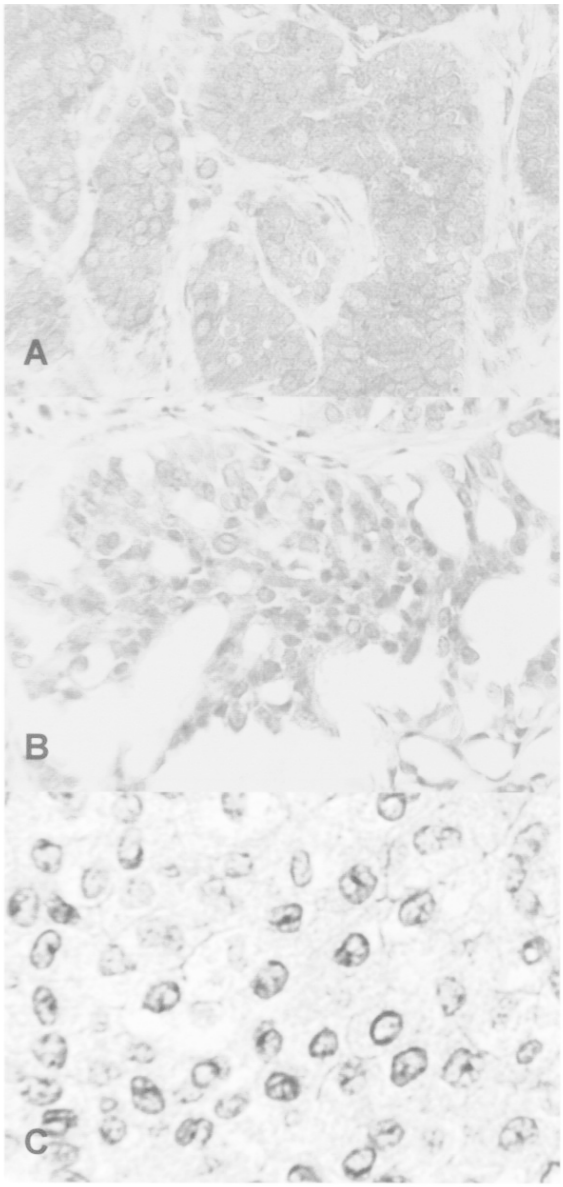

Figure 2. Immunohistochemical analysis of YB-1 and Pgp. (A), Cytoplasmic staining of YB-1. (B), Nuclear and cytoplasmic staining of YB-1. (C), Cytoplasmic and membrane staining of Pgp. (A and B, x250; C, x400; counterstained with haematoxylin).

samples exhibited nuclear localization of the protein (Fig. 2B). Approximately 2-10% of YB-1 positive cancer cells exhibited nuclear expression in each of the samples. Of the 36 samples for which peri-tumoral benign breast tissues were available, 29 (81%) exhibited YB-1 expression. The expression in such tissues was not associated with the immunoreactive score in the corresponding cancer sections, 43% of tumors exhibited Pgp immunopositivity (Fig. 2C).

Relationship of YB-1 and PgP immunoreactivity with prognostic variables. There was no significant association between YB-1 scores in breast cancer tissue and tumor size, lymph node metastasis, histologic grade and estrogen receptor status, when analyzed individually (Table II). However, we observed that the group of breast cancers with poorest prognostic characteristics (lymph node positive and estrogen receptor

Table II. YB-1 protein expression levels in different subgroups of breast cancers.

Factors	Total no. of patients	YB-1 median immunoreactive score
Patient's age (years)		
<50	43	5
>50	56	4
P-value		0.77
Primary tumor		
T1 and T2	88	4.5
T3 and T4	11	4
P-value		0.87
Regional lymph node metastasis[a]		
Negative	47	4
Positive	50	5
P-value		0.11
Estrogen receptor[b]		
Positive	64	4
Negative	33	5
P-value		0.14
Histological grade		
I and II	47	5
III	52	4
P-value		0.42

[a]Lymph node status for two other patients were not available. [b]Estrogen receptor status were not tested for two patients at diagnosis.

Table III. Increasing proportion of tumors with high YB-1 expression in breast cancers of poorer prognostic category.

	Prognostic category		
	Ln^-/ER^+	Ln^-/ER^- or Ln^+/ER^+	Ln^+/ER^-
No. of tumors	32	45	18
No. with high YB-1 score (6-15)	7 (22%)	16 (36%)	9 (50%)

Ln, lymph node; ER, estrogen receptor.

negative), had the highest proportion of breast cancers with high YB-1 expression (50%), compared to that of the group with intermediate prognosis (lymph node positive/estrogen receptor positive or lymph node negative/estrogen receptor negative; 36%) and that of the group with the best prognostic parameters (lymph node negative and estrogen receptor positive, 22%) (P=0.017) (Table III). On the other hand, Pgp expression did not show any correlation with any of the clinicopathological factors. There was no association detected between YB-1 scores with Pgp immunopositivity (P=0.369). Tumors with nuclear localization of YB-1 (5 in the group of tumors for which Pgp immunohistochemistry was performed) appeared to be Pgp positive (P=0.011), although 37% of tumors (19 of 52) without nuclear localization were Pgp positive as well.

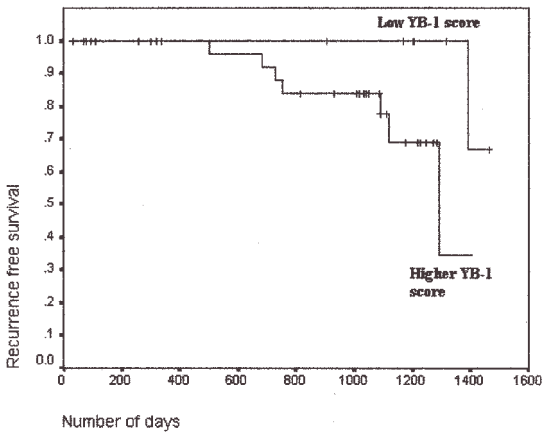

Figure 3. Higher expression levels of YB-1 protein (YB-1 scores 3-15) is associated with increased tumor recurrence in the group of breast cancer patients who did not receive adjuvant chemotherapy.

Association of YB-1 expression with recurrence and disease-free survival without adjuvant chemotherapy. Tumors with moderate to high YB-1 expression (scores 3-15) had a higher recurrence risk compared to those with low expression, even though the observation was not statistically significant (P=0.1718). In the group of 42 patients who did not receive adjuvant chemotherapy, only one of 8 patients (13%) with low expression (scores 0-2) of YB-1 in the primary tumor experienced disease recurrence, whilst 5 of 18 patients (28%) with high expression (scores 6 to 15) of YB-1 recurred. The difference in recurrence-free survival between the breast cancers of low YB-1 expression and that of higher (scores 3-15) expression was significant (P=0.034; Fig. 3) in this group of patients.

Association of YB-1 and Pgp expression with recurrence and disease free survival with adjuvant chemotherapy. YB-1 expression did not affect disease-free survival in the group of patients who received adjuvant chemotherapy (P=0.70; comparing 18 patients with low YB-1 score against 39 patients with moderate to high YB-1 score). However, among the 14 patients with high YB-1 expression (scores 6-15) and receiving chemotherapy, 2 of 7 (29%) receiving anthracycline-based chemotherapy recurred compared to no recurrence amongst patients receiving the CMF regime. While there was no difference in YB-1 score between all patients receiving CMF compared to anthracycline based regimes (P=0.961),

Table IV. YB-1 and Pgp status of breast cancer patients with recurrence after chemotherapy.

Patient's serial no.	Chemotherapy regime	YB-1 immuno-reactivity score	Pgp over-expression
23768	C	2	-
30071	C	2	-
23471	C	5	+
5204	C	4	+
31674	C	4	-
7840	A	5	+
21936	A	5	+
19001	A	10	-
16048	A	8	+

C, CMF chemotherapy. A, Anthracycline based chemotherapy.

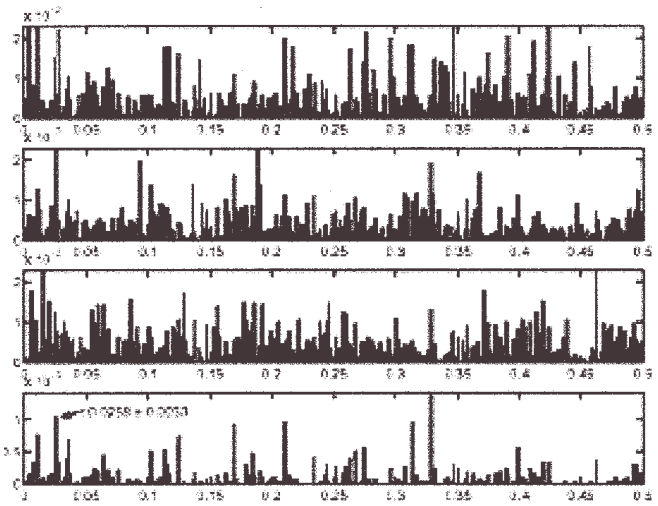

Figure 5. The RRM power spectra and the cross power spectrum of the three promoter sequences of MDR1 genes. The first three plots are the respective power spectra for the three promoters. The last plot is their cross power spectrum. One prominent peak appears at 0.0258±0.0033 in the last plot. The digital resolution is 0.0033.

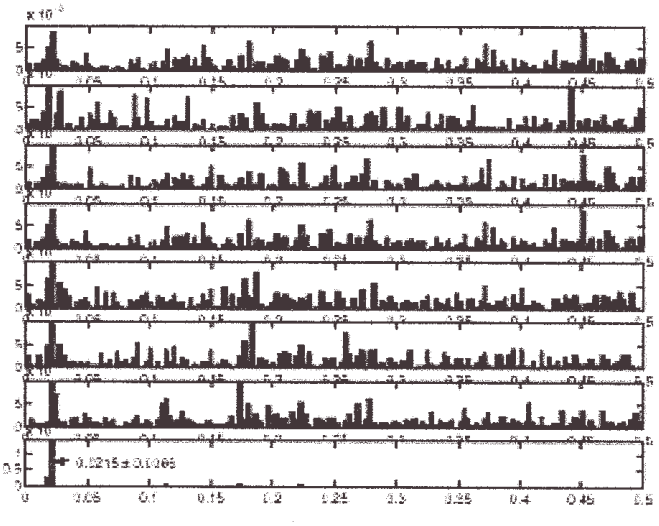

Figure 4. The RRM power spectra and the cross power spectrum of the seven proteins listed in Table I. The first seven plots are the power spectra for each of the seven YB-1 proteins. The last plot is the cross power spectra. The prominent peak around 0.0215±0.0066 is the characteristic frequency. The digital resolution was computed as 1/151=0:0066 since the length of the shortest protein is 151.

patients who developed recurrence despite being on the CMF regime, have a lower YB-1 score (range 2-5) compared to that of those patients receiving anthracycline-based therapy with recurrence (YB-1 score range 5-10; P=0.024) (Table IV). On the other hand, Pgp over-expression was not significantly associated with disease-free survival (P=0.34) or with recurrence after adjuvant chemotherapy (P=0.524).

Direct interaction of the YB-protein and promoter sequences of MDR1 genes. Fig. 4 shows the RRM power spectra and cross power spectrum of seven YB proteins. The first seven plots are the respective power spectra for each of the proteins. The last plot is the cross power spectra. There is a prominent peak around 0.0215±0.0066, which represents the characteristic frequency. According to the RRM theory, we may assume that 0.0215±0.0066 is the characteristic frequency representing the binding between YB and Y-box sequence, since the major common feature among the seven YB proteins is their ability to bind the Y-box DNA sequence. Similarly, the RRM power spectra and the cross power spectrum of the three promoter sequences of MDR1 genes are shown in Fig. 5. A prominent peak appears at 0.0258±0.0033 in the cross power spectrum (the last plot). For the sequences tested, the YB proteins and MDR1 promoters share a common characteristic frequency around 0.0215, suggesting a possible direct interaction between the protein and the promoter sequence.

Discussion

In this study, we have correlated YB-1 expression in breast cancer with established prognostic factors. We have shown that high YB-1 expression was associated with breast cancers of an aggressive phenotype (lymph node positive/hormone receptor negative). Differences in YB-1 expression resulted in a measurable effect on the clinical course of breast cancer. Without chemotherapy, primary breast cancers with higher YB-1 expression have a higher recurrent risk compared to those with lower expression. This result is consistent with a separate study involving primarily Caucasian breast cancer patients (21).

The significance of nuclear localization of YB-1 is yet unclear. Nuclear localization of YB-1 was found to affect prognosis in squamous cell lung cancer, ovarian serous adenocarcinoma and synovial sarcoma, but not in lung adenocarcinoma (25-27). It has also been reported that nuclear localization of the YB-1 protein was found to be associated with high levels of Pgp expression in human breast cancer (18) and osteosarcoma (15), but not in lung (13) and colorectal cancer (14). Similarly, we have found that

nuclear localization of YB-1 is associated with a propensity for Pgp expression. However, the proportion of breast cancers with nuclear localization of YB-1 appears to be low as evidenced by 11% in our present study and 13% of 86 patients in that reported by Janz *et al* (21) as compared to other cancers, such as non-small cell lung cancer (45% of 196 patients) (26) and ovarian cancer (45% of 35 patients) (28).

Using a computational approach based on the Resonant Recognition Model, we have also shown that direct binding between the YB-1 protein and the MDR1 gene promoter is indeed possible, further verifying experimental evidence that YB-1 is involved in the regulation of MDR1 gene in cancer cells (18,29). However, the present study also shows that the absence of nuclear localization of YB-1 does not necessarily preclude Pgp expression. In those tumor samples, it is possible that nuclear localization of YB-1 may not have been prominent enough to be detected by immunohistochemistry, or that other promoters are also operative (30).

The association between YB-1 and Pgp suggests that YB-1 may confer multi-drug resistance in breast cancer. Anthracyclines and taxanes are Pgp substrates and *in vitro* studies have shown that resistance to these drugs are associated with Pgp expression in breast cancer (31). On the other hand, there are clinical studies which did not show a better prognosis for patients with Pgp negative breast cancers (32,33). Our study has also shown a lack of correlation between disease-free survival and Pgp expression. Nevertheless, YB-1 may affect chemotherapy resistance in many other ways, possibly through DNA repair (2), or transcriptional or translational control of other proteins conferring drug resistance, even though there was no statistically significant difference in recurrence risk between patients with low YB-1 expression and higher YB-1 expression who received adjuvant chemotherapy.

However, it is interesting that although most of the patients received CMF chemotherapy, 80% of those patients who suffered tumor recurrence and had YB-1 scores of 5 or more, received anthracycline-based (e.g., Adriamycin) chemotherapy regimes. This suggests that YB-1 expression in breast cancer may possibly predict tumor resistance to different chemotherapy regimes, providing a marker that aids in the choice of adjuvant chemotherapy for breast cancers. As CMF and anthracycline-based regimes are mainstays of breast chemotherapy, larger clinical studies will be useful to verify if YB-1 expression would be useful in determining the choice of chemotherapeutic regimes for breast cancer patients.

Acknowledgements

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