A study on expression and genetic polymorphisms of Glutathione S-transferases and associated metabolic pathways in human breast cancers

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

A. SHIVA SREENATH

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046, India

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Enrolment No. 99LAPH07

Dedicated

To

My

Parents

University of Hyderabad

(A central university Established in 1974 by an act of parliament)

HYDERABAD – 500 046, INDIA

DECLARATION

I hereby state that the work embodied in this thesis entitled **"A study on expression and polymorphisms of Glutathione S-transferases and associated metabolic pathways in human breast cancers"** has been carried out by me under the supervision of Prof. P. Reddanna and that this has not been submitted for any degree or diploma of any other university earlier.

(Prof. P. Reddanna)
Research Supervisor

A. Shiva Sreenath

(A. Shiva Sreenath)
Research Student

University of Hyderabad

(A central university Established in 1974 by an act of parliament)
HYDERABAD – 500 046, INDIA

CERTIFICATE

This is to certify that Mr. A. Shiva Sreenath has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D. ordinance of this university. We recommend his thesis **"A study on expression and polymorphisms of Glutathione S-transferases and associated metabolic pathways in human breast cancers"** for submission for the degree of Doctor of Philosophy of this University.

Prof. P. Reddanna
Supervisor

Prof. P. REDDANNA
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046, India.

Head
Dept. of Animal Sciences

Head
Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046, India.

Dean
School of Life Sciences

26/8/03

DEAN

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Abbreviations

BCIP	:	5-bromo-4-chloro-3-indolyl phosphate
cm	:	centimetre
CuOOH	:	cumene hyderoperoxide
CDNB	:	1-chloro-2,4-dinotrobenzene
DCNB	:	1,2-dichloro-4-nitrobenzene
DNA	:	deoxy ribonucleic acid
EA	:	ethacrynic acid
EDTA	:	ethylene diamine tetraacetic acid
GSH	:	Glutathione reduced
GST	:	Glutathione S-transferases
g	:	gram
h	:	hour
IgG	:	immunoglobulin
kDa	:	kilo dalton
min	:	minute
mM	:	milli molar
Mol. Wt	:	molecular weight
MDR	:	multidrug resistance
M	:	molar
n	:	nano
nm	:	nano meter
NBT	:	nitro blue tetrazolium
PAHs	:	polycyclic aromatic hydrocarbons
s	:	second
SDS-PAGE	:	sodium dodecyl sulphate polyacylamide gel electrophoresis
Tris	:	tri (hydroxyl methyl) aminomethane
UV-VIS	:	ultraviolet-visible
μ	:	micro
Δ ε /mM/cm	:	molar extinction coefficient
λ max	:	lambda maximum

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A. Shiva Sreenath

INTRODUCTION

Introduction

In most of the cancer cases the environmental carcinogens, cigarette smoke or the betel nut in addition to other factors may be operating in concert with the obvious factors. In spite of the fact that there seems to be a direct cause and effect relationship between the cancer-causing factors (carcinogens) and the cancer development, the process may be somewhat more complex. With many carcinogens another agent is needed to act along with the primary stimulus in order to produce a malignant lesion. There are two schools of thought suggesting the theory of cancer initiation. One, the somatic mutation theory and the other aberrant differentiation theory. In most of the cancers there is an accumulation of chromosomal defects that cannot be corrected along with reduced repair ability in the older persons. A defect any where in the process of cell differentiation could lead to malignancy. Environmental influences in the broadest sense probably are related to the development of most cancers. However, majority of these environmental causes are thought to be related to behavioural factors (Eg. Cigarette smoking, alcohol and delayed child bearing), viral agents (Eg. Human papilloma virus and Epstein barr virus), occupational exposures (Eg. Benzene, asbestos and coke oven emissions), or dietary factors (Eg. animal fats and aflatoxins). Other factors are drinking water contaminants, passive exposure to environmental tobacco smoke, non-occupational exposures to asbestos, indoor radon, ultraviolet radiation and electric and magnetic fields. Air pollution and hormonally active aromatic organochlorines are considered related to human cancers.

Epidemiological studies present conflicting evidences regarding the association of aromatic organochlorines and breast and uterine cancers (Adami, 1995).

Some of the environmental risk factors for development of breast cancer identified in recent studies are:

1. Organochlorine compounds including pesticides such as dichloro diphenyl trichloro ethane (DDT), dichloro diphenyl ethane (DDE) and chlordane

2. Polychlorinated biphenyles (PCB), which are used as electric insulators.

3. Polycyclic aromatic hydrocarbons (PAHs) formed during the incomplete burning of organic material, found in car exhaust, cigarette smoke and charcoal broiled food.

4. Electromagnetic field – generated by the flow of electricity overhead electric powerlines and generators found in power plants are a source of cancers.

Many organochlorines are capable of binding to estrogen receptors and are hormonally active in animals causing either estrogenic or antiestrogenic effects. However, the observed hormonal effects are 3 orders of magnitude less than with naturally occurring estrogens. In human studies, some evidence suggests that women exposed to higher levels of dichlorodiphenyl ethane (DDE) have

decreased duration of lactation. These hormonal properties are of interest since estrogens are growth factors for tissues of the breast and uterus. At lower doses, the aromatic organochlorines may serve as tumor promoters to increase the rate by which a transformed cell grows into a clinically detectable tumor.

Several studies have suggested that exposure to these chemicals may affect estrogen production. Under laboratory conditions, they have been found to increase mammary tumors in animals, and this may indicate an increase in the risk of breast cancer.

Breast Cancer is one of the major cancers in women all over the world. Breast cancer occurrence is on raise and is the second most common cancer among women in India. The number of women being affected by breast cancer is increasing especially in the major cities of India. In India the average incidence rate of breast cancer is 16 per 100,000, varying from 22-28 per 100,000 in urban settings to 6 per 100,000 in rural areas. Breast and cervical cancer are among the common cancers in women in India and according to the data collected by the national cancer registry program (1981-2001) of the ICMR, breast cancer is the highest among all sites of cancers in the national capital. The incidence of breast cancer in Delhi is at 21.3 percent, the highest among all types of cancers in women. A life time risk of developing breast cancer in Mumbai city has increased to 1 in 28. The cause of increasing incidence is not known, however, there is a suspicion that it is related to Western life-style. It is a heterogenous disease of

middle age, which grows at very different rates in different patients and is often a systemic disease at the time of initial diagnosis. Mortality seems to increase with age and is double in women whose mothers or sisters have had the disease. The incidence of breast cancer in the US women is approximately five times that in Asian women and is predominantly post menopausal in its age distribution.

The other risk factors for the development of breast cancers.

- Family history: Incidence and mortality seems to increase with age and is double in women whose mothers or sisters have had the disease.

- Unmarried women have 40% higher rate of breast cancer incidence than married women and late pregnancy also seems to increase the risk of breast cancer.

- Nulliparity: Individuals having no children are at higher risk of developing breast cancer compared to those having children. Parous women have significantly better 5-year survival rate (60%) compared to those of nulliparous (46%).

- Early onset of menarche (approximately 11 years) adversely affects survival. Menarche at age 15 years is said to be optimal.

- Late menopause is also a risk factor of breast cancer due to prolonged exposure of the body to estrogens.

- Cystic breast disease: This is usually clinical presentation of small mass that appears to grow rapidly. Despite its rapid growth, it does not invade the skin or ulcerate. Diagnosis is established by biopsy.

- Obesity: Obesity increases the estrogen levels in postmenopausal women as a result of increased aromatization of androgen to estrogens in excess fat deposits (Zumoff, 1982). Obesity is also associated with decreased levels of sex hormone-binding globulin leading to a raise in bioavailable levels of both estradiol and testosterone (Peiris, 1989; Preziosi, 1993). Because the affinity of the globulin is greater for testosterone than for estradiol, the androgen/estrogen balance shifts towards androgen. This is thought to directly deposit fat to the abdomen rather than to the femoral gluteal region (Evans, 1983).

Clinical diagnosis:

Mammogram: This is a soft tissue x-ray which may detect the presence of a cancer. Nil on palpation can prove to be a carcinoma on a mammogram. The tumor detected by a mammogram is represented in figure I.1a.

Thermogram: Infrared scanner is used to measure heat emissions. Abnormal patterns will be produced as a result of increased vascularization in the tumor area.

Xenoradiography: X-ray films of the breast are taken using an electrically charged selenium coated plate in place of photographic film.

Aspiration biopsy: In this procedure a tissue sample from the breast lump is taken using a syringe and needle.

Excision Biopsy: A small piece of tissue is removed surgically and is then examined either as a frozen section or as a paraffin section.

Lumpectomy: This entails surgical removal of the lump only and may be used in the treatment of medial tumors.

Partial mastectomy: The tumor is surgically removed with at least two centimetres of healthy tissue surrounding it.

Subcutaneous mastectomy: The breast tissue is removed but the skin and nipple are left intact and prosthesis is implanted.

Simple mastectomy: The whole breast is removed and there may be simple excision of the lower axillary nodes.

Modified radical mastectomy: The whole breast is removed and there is a total clearance of adjacent axillary lymph nodes.

Radical mastectomy: The whole breast and adjacent lymph nodes are removed together with the pectoralis major and minor muscles. Mastectomized tissue is shown in figure 1b and 1c.

General biochemical markers:

The expression of estrogen receptor (ER) proteins in normal breast epithelium is associated with increased breast cancer risk and may reflect abnormality and increased responsiveness to estrogen (Khan, 1997). Higher proliferative activity is found in ER positive than in ER negative specimens of ductal hyperplasia with atypia but the reverse is seen in ductal carcinoma insitu (DCIS) lesions and invasive breast cancer (Schmidt, 1995). The role of ER in breast cancer development is thus unclear, but it may be influenced by paracrine activity of growth factors (Bernstein, 1998).

Expression of the onco proteins HER-2 neu in DCIS lesions increases with increasing grade of malignancy (Leal, 1995; Albonico, 1996) and the presence of extensive necrosis in DCIS is associated with the expression of mutated p53 and HER–2 neu (Bodis, 1996; Bobrow, 1995). In addition to this, high S-phase on flow cytometry is a marker for early recurrence and early death. The biochemical markers of progressive carcinogenesis, including oncogene, growth factors and

Human breast tumor

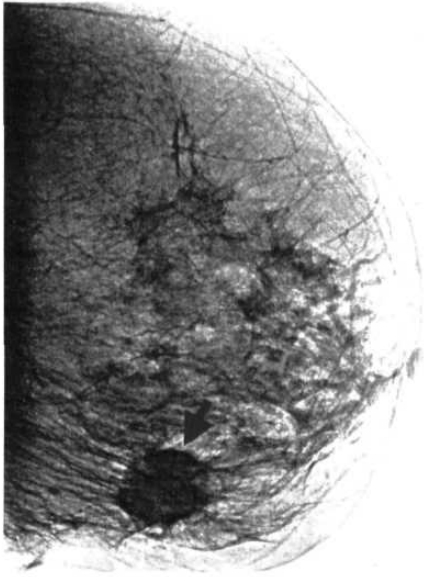


Fig.I. 1a

Mammogram showing the tumor in the breast as a thick mass with dense vasculature

Mastectomy

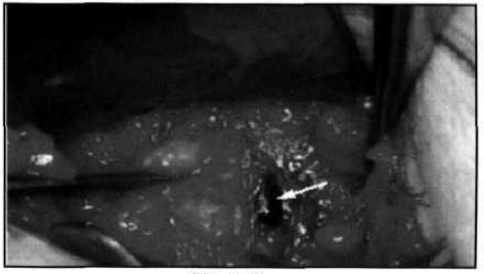


Fig. I.1b

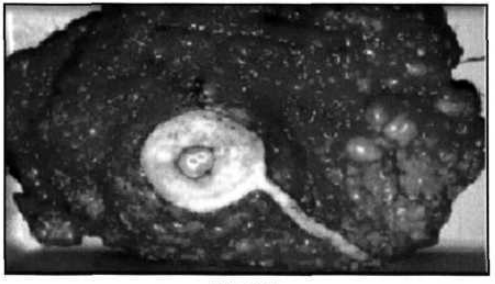


Fig. I. 1c

A. Nodal involvement shown in blue

B. Mastectomized tissues showing the affected tissue

steroid receptors, have recently been quantified in premalignant lesions (Albonico, 1998; Querzoli, 1998).

Among the existing biomarkers, GSTs can form a better biochemical marker which can be employed in assessing high risk individuals and early diagnosis. Initiation of carcinogenesis is the process of irreversibly committed conversion of individual cell into a preneoplastic cell wherein the metabolic pathways are distorted for the prevalence of conversion. The subsequent step is the progression stage where the cellular reproductive potential is immensely increased at the cost of the neighbouring cells. Molecular phenomenon of initiation of carcinogenesis is the covalent modification of DNA structure by the exogenous and/or endogenous harmful molecules which are electrophilic. Glutathione S-transferases (GSTs) being the sequestering enzymes of electrophilic molecules, they are involved in the prevention of initiation of carcinogenesis. In preneoplastic and neoplastic cells it is observed that higher levels of specific isoforms of GSTs are expressed and this is dependent on the tissue in which it is taking place.

Glutathione S-transferases (EC 2.5.1.18) are a family of structurally related multifunctional enzymes found in organisms living in aerobic environment (Mannervik, 1985). These enzymes catalyze reactions between the tripeptide glutathione (ϒ-Glu-Cys-Gly; GSH) and a second electrophilic compound. A broad spectrum of substrates and different types of reaction mechanisms have been identified for GSTs. Among other activities, these enzymes catalyze the first

step in the formation of mercapturic acids (N- acetylcystein derivatives) (Fig. I.2).

The detoxification enzymes are broadly classified into two, phase I and phase II systems. Phase I system includes Cytochrome P_{450} monoxygenases while GSTs belong to phase II category (Fig. I.3).

Evolution of GSTs

Drug detoxification enzymes have existed in both prokaryotes and eukaryotes for more than 2.5 billion years (Nebert, 1994 & 2000). GSTs constitute a very ancient protein superfamily that is thought to have evolved from a thioredoxin-like ancestor in response to the development of oxidative stress (Martin, 1995; Koonin, 1994). Other GSH and cysteine binding proteins share the thioredoxin-like fold (Martin, 1995) and it is increasingly becoming clear that GSTs share sequence and structural similiarities with several stress-related proteins in a wide range of organisms (Rossjohn, 1996). It is thought that multiple GST classes arose by a process of gene amplification followed by divergence, perhaps involving a mechanism similar to DNA shuffling, resulting in novel catalytic activities (Armstrong, 1997; Hansson, 1999). Many workers have used sequence comparisons to generate phylogenetic trees to identify likely patterns of divergence. Ideally, it ought to be possible to compare all full-length sequences known to code for GSTs, but in practice a subset of sequences is usually used to avoid misleading results (Snyder, 1997). In making alignments, therefore, it is necessary to select a subgroup of GSTs, and this accounts for the slight

Fig. I. 2. Mercapturic Acid Pathway

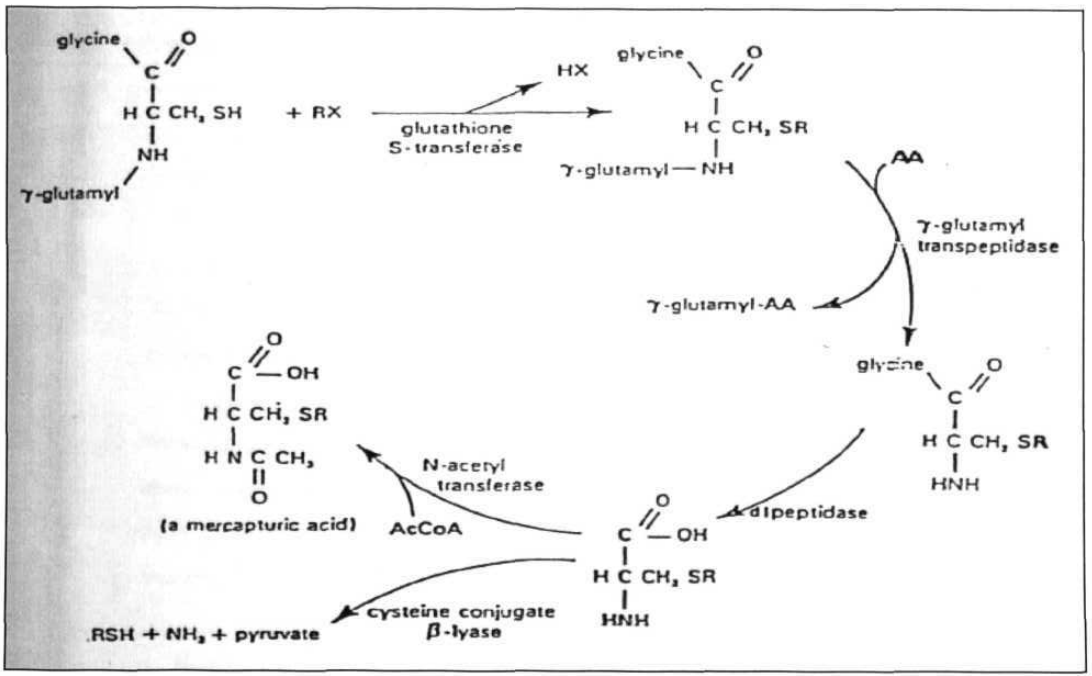
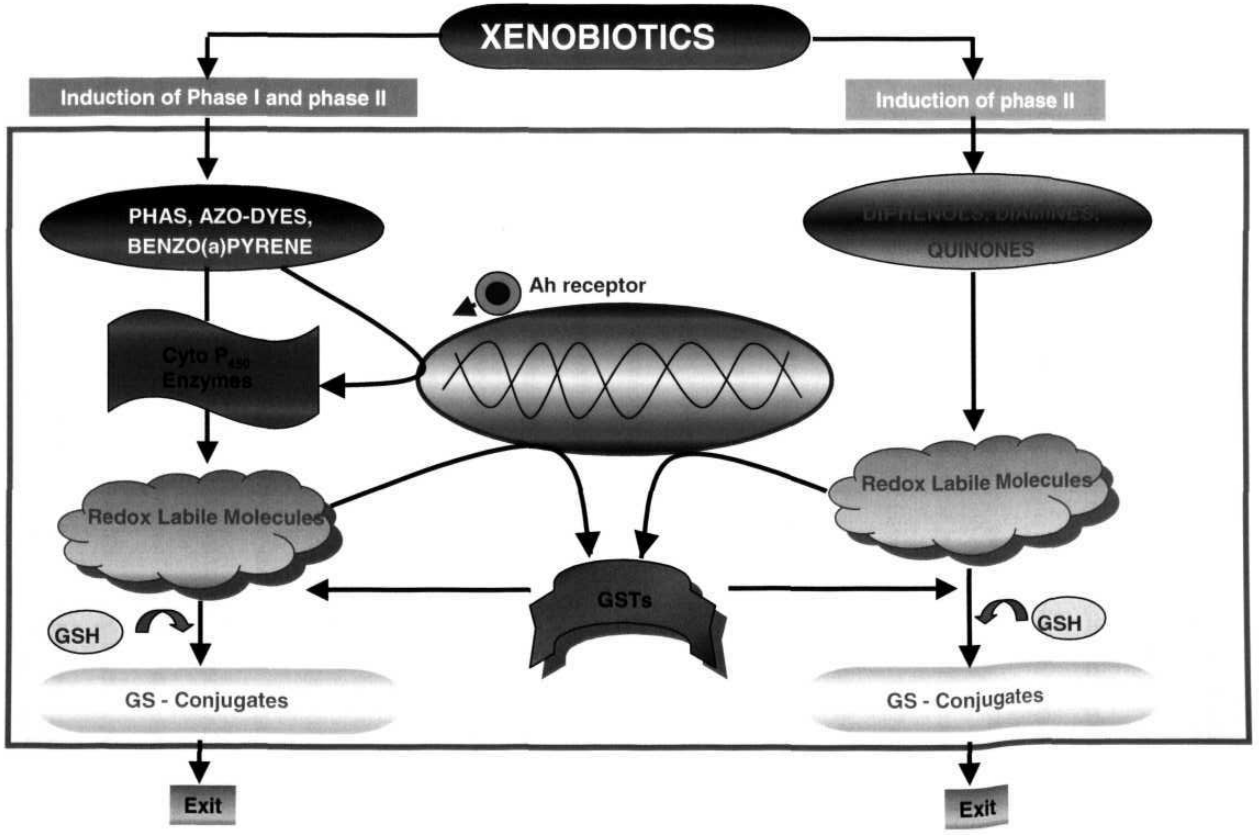


Fig.I. 3. Xenobiotic detoxification by phase I and phase II enzymes



differences, for example, between trees published earlier (Board, 2000). Protein families arise as a result of duplication and divergence of entire coding regions, independently folding domains or short sequence motifs that can be inherited in proteins with sometimes quite different functions to the primordial ancestor (Henikoff, 1997). As pointed out in the discussion of individual GST classes, several non-GST proteins have been implicated as being related to GSTs by sequence alignment (Rossjohn, 1996) or based on immunoblotting (Blocki, 1993). Examples include bacterial stringent starvation proteins, plant pathogen stress resistance proteins, the URE2 protein from *S. cerevisiae* (Rossjohn, 1996) and eukaryotic translation elongation factor 1Υ (Koonin, 1994; Blocki, 1993 & 1992). These relationships suggest that a common stress related ancestor may have pre-dated the evolution of the thioredoxin fold. In cases where only a small number of GST classes have been described, it is possible that other classes exist which have not yet been discovered and which may carry out novel functions not necessarily associated with detoxification.

Classification and occurrence of GSTs

The cytosolic GSTs have been divided into a number of structurally distinct classes - Alpha, Mu, Pi, Theta, Kappa, Sigma, Zeta on the basis of sequence similarities (Mannervik, 1985), and their chain-fold topology is essentially the same with the exception of the C-terminus of the enzyme. Arabic numerals are employed for numbering each of the separate genes. A class may contain several isoenzymes, with subunits numbered in the order that they were

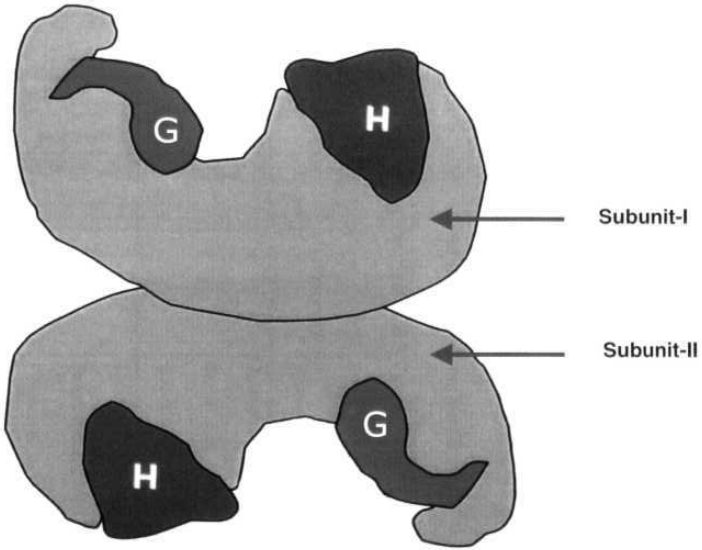
discovered. The enzymes are dimeric and the first human Mu class subunits are therefore called hGST M1-1 to indicate that it is a dimer of subunit 1 (Mannervik, 1992). Each subunit consists of two domains. Domain one also called G-site is a smaller one for the binding of GSH and the second domain also called H-site is a larger one for the binding of various electrophilic compounds (Fig. I. 4).

In addition to cytosolic GSTs, at least two membrane bound GSTs exist in mammals. These are referred to as microsomal GSTs and leukotriene C_4 synthases (LTC_4S). The microsomal enzymes are involved in the detoxification of xenobiotics, where as LTC_4S, as the name indicates, conjugate GSH to leukotriene A_4 to generate Leukotriene C_4.

GSTs are widely distributed in nature, found in bacteria, yeast, molds, fungi and molluscs. Essentially, all eukaryotic species appear to possess multiple isoenzymes. The various isoforms of this enzyme are species, sex, tissue, and age specific. The human liver GSTs mostly belong to the classes alpha, mu, and pi. Majority of human tumors and human tumor cell lines express significant amount of class pi GST.

The distribution of GSTs has been studied in extrahepatic organs, including kidney (Tateoka, 1987; Singh, 1987), lung (Koskelo, 1981), brain (Theodore, 1985), skin (Del Boccio, 1987), intestine (Peters, 1989), adrenal gland (Sherman, 1983), testis (Aceto, 1989), prostate (Tew, 1987), heart (Hirrell, 1987), blood vessels (Tsuchida, 1997), and skeletal muscle (Singh, 1991).

Fig. I. 4. Model of dimeric glutathione S-transferase demonstrating its two active sites



GST dimer showing complete active site in each individual subunit, including a subsite for glutathione (G-site) as well as a subsite for the elecltrophilic substrate (H-site) (Mannervik et al., 1985)

Gene families encoding cytosolic GSTs

A large number of cytosolic GST isozymes have been purified from rat and human organs based on their primary structure. In comparison if the primary structure is more than 40% identical they are included in the same class. If the homology is less than 30% then they are assigned to a separate class.

The hypothesis that each class represents a separate gene family is supported by the distinct structure of their chromosome localization.

Table I.1: Gene families encoding cytosolic GSTs

Class	Source	Size in Kb	# of Exons	% Homoglogy	References
Alpha	Rats Mice Humans	11 to 12	7	55	Telakowski et al., 1986 Daniel et al., 1987 Suzuki et al., 1983
Mu	Rats Mice Humans	5 to 6	8	65	Lia et al., 1988 Reinhurt et al., 1993 Pearson et al., 1993 Fan et al., 1992
Pi	Rats Mice Humans	3	7	?	Okuda et al., 1987 Bammler et al., 1994 Cowell et al., 1988
Theta	Rats	4	5	50	Ogura et al., 1994

Substrates catalyzed by GSTs

The reactions catalysed by GSTs can be broadly classified as conjugation reaction, oxidation-reduction reaction and isomerization reaction. In each of these reactions GSH is a nucleophilic reactant towards an electrophilic substrate (Mannervick, 1986) (Fig. I. 5).

Conjugation reactions and detoxification

The major role of GSTs is the detoxification of exogenous xenobiotics and their metabolites and endogenous toxic compounds in the phase II reaction of drug detoxification pathway. They conjugate the electrophilic centre of toxic, hydrophobic compounds to the sulfur atoms of GSH and the nature of the reaction is nucleophilic (Fig. I. 6). The resultant water soluble S-conjugate is processed and eliminated via the classical mercapturate pathway (Boyland, 1969, Chasseaud, 1979).

Some substrates, such as chloronitrobenzenes, which undergo nucleophilic aromatic substitution reactions, have been analyzed rigorously to understand the mechanisms of catalysis (Graminski, 1989). Other substrates appear biologically relevant, for example, hydroxyalkenals formed by oxidation of membrane lipids (Hubatsch, 1998) and orthoquinones derived from catecholamines, Eg: dopamine and epinephrine (Baez, 1997). O-quinone aminochrome is an oxidation product of the neurotransmitter dopamine and can participate in redox cycling, giving rise to toxic oxygen species (Segura-Aguilar

1989). This process, when taking place in substantia nigra cells of the brain, is believed to contribute to the development of Parkinsons disease (Baez, 1995). The conjugation of aminochrome with GSH, catalyzed by GSTs, competes with the redox cycling. Thus GSTs play a physiologically important protective role in cellular systems (Segura-Aguilar, 1997).

There are also pharmaceuticals that are substrates for GSTs, such as ethacrynic acid, which has been tested as a competitive substrate for GSTs with the goal of suppressing tumor cell resistance to certain cytostatic drugs (O'Dwyer, 1991). Another example is nitrosocimetidin, a carcinogenic metabolite of the drug cimetidine used in treatment of intestinal disorders (Jensen & Stelman, 1987). Studies on these compounds have shown that GSTs can catalyze the denitrosation of the toxic substrates, forming less harmful guanidine derivatives and S-nitroso-glutathione (Jensen, 1997). GST isoenzymes display distinct substrate selectivities, for example two structurally related human isoenzymes, hGST M1-1 and hGST M2-2, exhibit large differences in activity with the substrates aminochrome and cyanoDMNG (Mannervik, 1992). Human GST M2-2 is about 100 times more active than hGST M1-1 with the above mentioned substrates, which are referred to as hGST M2-2 characteristic substrates. Human GST M2-2 also has a 100-fold higher activity with 1,2-dichloro-4-nitrobenzene (DCNB), which undergoes a nucleophilic aromatic substitution reaction. This compound is structurally related to 1-chloro-2, 4-dinitrobenzene (CDNB), which is

Fig. I. 5. Catalytic and non-catalytic functions of GSTs

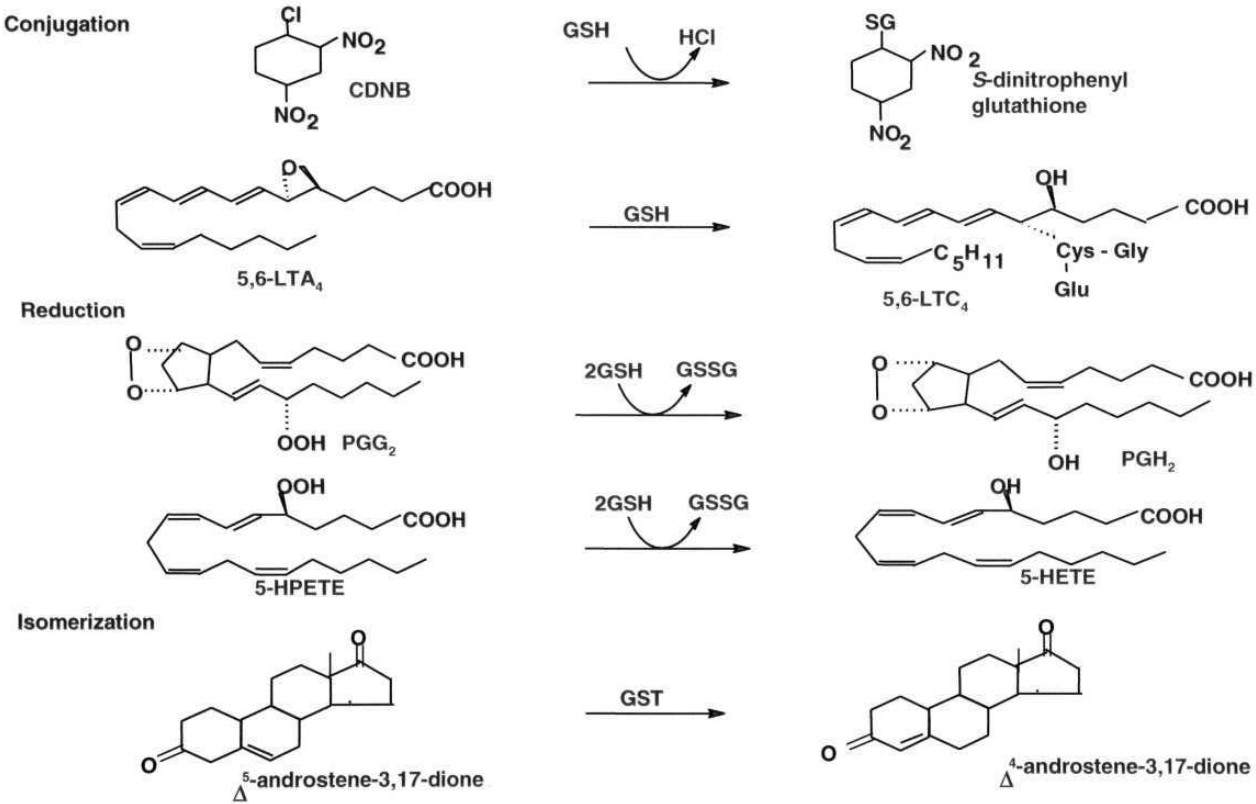
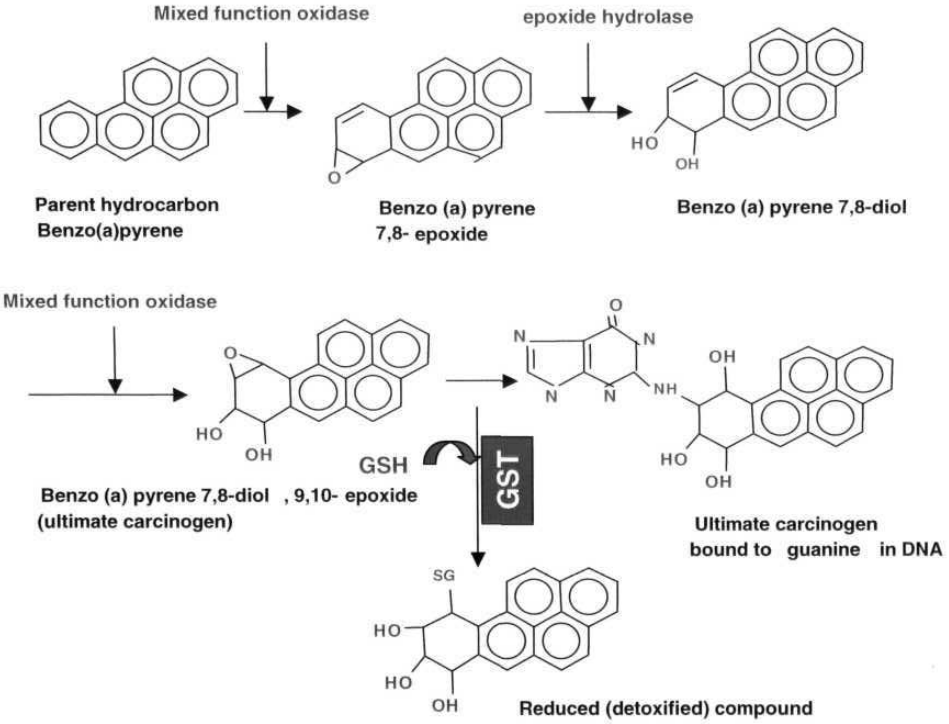


Fig. I. 6. Mechanism of detoxification of benzo(a)pyrene by GSTs



the most frequently used substrate in GST activity assays, but the two Mu class GSTs do not show any major differences with CDNB.

Gutathione conjugation reaction and toxification

A potentially more serious situation can arise with a small number of GST substrates that yield a GSH conjugate, or a metabolite of the conjugate, that is more reactive than the parental compound (Keen, 1978). These groups of compounds have been referred to as directly acting toxic GSH conjugates and indirectly acting toxic GSH conjugates. Incomplete detoxification by GSTs occur with certain esters, ethers and organic phosphates when conjugation leads to cleavage of the substrate with only one of the two products being conjugated. This process has been called thiolysis (Keen, 1978) and in the case of *p*-nitrophenol acetate, the herbicide fluorodifen, and the insecticide EPN, it results n the release of *p*-nitrophenol; presumably, the *p*-nitrophenol is metabolized by UDP-glucuronosyl transferase and phenol sulfotransferase. Thiolysis represents incomplete detoxification because the unconjugated cleavage product still provides a chemical threat to the cell. Although toxification by GSTs is undesirable in normal circumstances, it can be exploited in cancer therapy to treat tumors that overexpress GSTs. For example, drugs that either yield directly acting toxic conjugates or are cleaved by GST to produce toxic metabolites, may be of value in targeting certain cancers. Nitrogen mustards have been synthesized that, when cleaved by GST, liberate a cytotoxic phosphate moiety (Lyttle, 1994).

Peroxidase activity of GSTs

GST isoenzymes also exhibit glutathione peroxidase activity and catalyze the reduction of organic hydroperoxides to their corresponding alcohols. This type of reaction is thought to represent nucleophilic attack by GSH on electrophilic oxygen. The substrates that GST reduces include fatty acids, phospholipids, and DNA hydroperoxides. As these compounds are generated by lipid peroxidation and cause oxidative damage to DNA, it has been proposed that GSTs, as well as other GSH-dependant enzymes, help combat oxidative stress. Detoxification of lipid hydroperoxides by microsomal GSTs can occur *in situ* whereas detoxification of lipid hydroperoxides by cytosolic GST requires prior release of fatty acid hydroperoxides by phospholipase A2.

1. $ROOH + GSH \longrightarrow ROH + [GSOH]$
2. $[GSOH] + GSH \longrightarrow GSSG + H_20$

Isomerase activity

Several GSTs can catalyze the cis-trans isomerization of maleyl acetone to fumaryl acetone and maleyl acetic acid to fumaryl acetoacetic acid. An even small number of GST isozymes possess keto steroid activity and catalyze the conversion of Δ^5- 3-ketosteroids to Δ^4-3-ketosteroids (Fig. I. 5).

Multidrug resistance

Increased activity of GSTs has been implicated as a significant factor in acquired resistance to certain anticancer drugs (Hayes and Pulford, 1995). It was

shown that GST pi isoform expresses strongly in preneoplastic and neoplastic cells (Sato, 1989).

One isoform of class Pi GST termed hGSTP1-1 with molecular weight of 24.5kDa has a unique feature in that it gets post-translationally modified with glycosylation at the N-terminal end of the protein. Koo and his group (Koo, 1994) have clearly shown that this isoform has a multilevel regulation, as there is no quantitative compatibility with the transcript, protein and enzyme activity. In most of the cancers, class pi GST is the major form expressed, Eg. Colon, stomach, esophagus, uterine cervix, lung, etc. In humans it was shown that methylation of the cytidine residues in the promoter sequence of GST P1 is involved in the regulation of expression. Hypomethylation resulted in increased promotion and the hypermethylation in decreased promotion of expression (Lee, 1994). Apart from this, insulin increases the expression through a cis-acting element in the intron 1 of GST pi gene (Xia, 1993). The detection of this enzyme (pi class GST) facilitates the analysis of cancer progression and provides the basis for new methods of screening for carcinogens and carcinogenic modifiers.

GSTs as markers for cancer susceptibility

To understand the polymorphic nature of GST gene in a population various studies were undertaken (Board, 1981 and Windersten, 1991). Several studies have implicated GSTM1 gene deficiency to susceptibility of individuals to a range of cancers (Hayes and Pulford, 1995). Molecular epidemiology studies conducted in different geographical areas to monitor the GSTs expression revealed that about

50% of Caucasian population is devoid of GSTM1 gene. This lack of mu gene as a result of gene deletion was attributed to the increased risk of lung cancer development in the population (Board, 1981). Smoking and GSTM1 null genotype were found to be risk factors for squamous cell carcinoma (Bernadette Sehoket, 1998). Homozygous loss of GSTM1 gene has been suggested as a possible marker for high susceptibility to lung cancer among smokers. A hereditary difference in the expression of this form is due to deletion of the gene (Seidegard, 1988). The GSTM1 homozygous null genotype was associated with an increased risk of developing breast cancer, principally in association with postmenopausal breast cancer (Helzlsouer, 1998). It was suggested that glutathione and glutathione dependent enzymes play crucial role in tobacco-related tumorigenesis and may be considered as markers of carcinogen exposure. The prevalence of GSTM1 and GSTT1 null genotypes in oral premalignant leukoplakia cases and controls were ascertained using PCR technique (Urmila Nair, 1999). The risk of breast cancer increased as number of putative high-risk genotypes increased for a combined genotype GSTM1 null and GSTP1 valine heterozygosity (Watson, 1998).

Thus the molecular epidemiology studies on GSTs have revealed higher frequency of GST gene deletions in populations linked to higher susceptibility to variety of diseases (Chenevix-Trench, 1995). These studies on GST gene deletions are thus important to identify the high risk individuals in population.

Hence a thorough screening of population for cancer susceptibility at genetic level is required for prevention and efficient treatment of various types of cancers.

Scope of the present study

In view of the key role played by GSH and its metabolizing enzymes in the pathogenic conditions such as cancers, the present study is undertaken to study the status of GSH metabolism in breast cancer patients. The work is also focussed on studying concentration of glutathione and activities of various enzymes involved in glutathione metabolism. A special emphasis is given for purification and characterization of GSTs from breast cancer tissues. The study includes the expression of GSTs at both protein and mRNA levels. A case-control study on GST gene deletions in breast cancer patients and age matched controls was undertaken to assess the correlation, if any, between the two.

METHODOLOGY

Tissue Samples

Cancer and the adjoining normal tissues and blood samples from cancer patients were obtained from Mehdi Nawaz Jung (MNJ) Cancer Hospital, Red Hills, Hyderabad and Indo-American cancer hospital, Banjara Hills, Hyderabad.

Preparation of tissue homogenate

Normal and cancer tissues were homogenized in 50mM phosphate buffer, pH 7.0 containing 250mM sucrose, 1mM EDTA and 1mM PMSF using a glass homogenizer. Homogenization was done by keeping the glass homogenizer in an ice jacket and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheese cloth and then centrifuged at 10,000 X g at 4°C for 30 min. The supernatant was recentrifuged at 1,05,000 X g for 1h and the resultant supernatant was referred as the cytosolic fraction of the enzyme source.

Glutathione estimation

Reduced glutathione (GSH) estimation

GSH in normal and cancer tissues was estimated according to the method described by Paul and Russell (1976). According to this method, 250mg tissue was homogenized in 3.5ml of 100mM phosphate buffer pH 7.0 with 1ml of 25% phosphoric acid. This was centrifuged at 4°C for 30 min at 10,000 rpm. The supernatant was collected and 0.5ml was added to 4.5 ml of 100mM phosphate

buffer pH 7.0. This was used as the source for GSH estimation. The reaction mixture consisted of 1800µl of 100mM phosphate buffer pH 7.0, 100µl of tissue homogenate and 100µl of O-pthalaldehyde (OPT) (100µg). This reaction mixture was incubated at room temperature for 15 min and the emission of fluorescence was monitored at 420nm with excitation at 350nm on fluorescence spectrophotometer.

Oxidized glutathione (GSSG) estimation

Estimation of GSSG was done according to the method described by Paul and Russell (1976). According to this method the tissue was processed the same way as that for the estimation of GSH. 0.5ml of the undiluted tissue homogenate was incubated with 200µl of 40nM N-Ethyl Malaemide (NEM) for 30 min. To this 4.3ml of 0.1N sodium hydroxide was added. This was used as the source to estimate GSSG. The reaction mixture consisting of 1800µl of 0.1N sodium hydroxide, 100µl of tissue homogenate and 100µl of OPT (100µg) was incubated at room temperature for 15 min and the emission of fluorescence was measured at 420nm with excitation at 350nm on fluorescence spectrophotometer.

Different concentrations of GSH and GSSG were used as standards.

Glutathione S-transferase (GST) (2.5.1.18) activity assay

One unit of GST activity was defined as the amount of enzyme that catalyzes the formation of one micromole of S-2, 4-dinitrophenyl glutathione per min at 30°C in a reaction mixture containing 1mM GSH, 1mM CDNB in 100mM potassium phosphate buffer (pH 6.5) (Habig, 1981). The reaction was monitored spectrophotometrically at 340nm. Extinction coefficient of CDNB was taken as 9.6 $mM^{-1}cm^{-1}$.

CDNB + GSH ⟶ S-2, 4 dinitrophenyl glutathione + H^+ + Cl^-

Activity was calculated according to the following equation

$$\frac{\text{Difference in absorbance for 1min} \times \text{volume of the reaction mixture in ml}}{\varepsilon_{CDNB}\ (9.6) \times \text{volume of the enzyme in ml}}$$

Specific activity is expressed as units per mg of protein

Glutathione reductase (GR) (1.6.4.2) activity assay

GR activity was estimated according to the method described by Carlberg and Mannervik (1975). According to this method a unit of enzymatic activity is defined as the amount of enzyme that catalyzes the oxidation of 1nmol of NADPH in a reaction mixture containing 200mM phosphate buffer (pH 7.0) with 2mM EDTA, 2mM NADPH in 10mM Tris buffer pH 7.0, and 20mM GSSG. The reaction was initiated by the addition of 50µl of enzyme sample and the oxidation of

NADPH was recorded as decrease in absorbance at 340nm for 60sec. Extinction coefficient of NADPH was taken as $6.2mM^{-1}\ cm^{-1}$.

Specific activity is expressed as units per mg of protein, where one unit is defined as one nmole of NADPH oxidized per min.

$$NADPH + H^{+} + G\text{-}S\text{-}S\text{-}G \longrightarrow NADP+2GSH$$

Activity of enzyme was calculated according to the following equation

$$\frac{\text{Difference in absorbance for 1min} \times \text{volume of the reaction mixture in ml}}{\varepsilon_{NADPH}\ (6.2) \times \text{volume of the enzyme in ml}}$$

Glutathione peroxidases (GPx) activity

GPx activity was estimated according to the method described by Flohe and Gunzler (1986), and Wendel, (1986). According to the method perfused tissues were homogenized (10%w/v) in 50mM phosphate buffer, pH 7.0, containing 1mM EDTA, 1mM PMSF and 250mM sucrose. The cytosolic fraction was used as the enzyme source to estimate the peroxidase activity. The activity was measured by monitoring the oxidation of NADPH at 340 nm in a reaction mixture containing 50µl of enzyme, 250mM phosphate buffer pH 7.0, 2.5mM EDTA, 2.5mM sodium azide, 1mM GSH, 2mM NADPH, one unit of GR incubated for 5 min at room temperature. Total GSH Px activity was assayed by using 1.5mM cumene hydroperoxide. The Se-dependent GSH Px activity was assayed, by using 12mM hydrogen peroxide. Non-Se-dependent GSH Px activity was

calculated by substracting Se-dependent-GSH Px activity from the total peroxidase activity (Reddy, 1981).

$$ROOH + 2GSH \longrightarrow ROH + H_2O + G\text{-}S\text{-}S\text{-}G$$

Activity of enzyme was calculated according to the following equation:

$$\frac{\text{Difference in absorbance for 1min} \times \text{volume of the reaction mixture in ml}}{\varepsilon_{NADPH}\ (6.2) \times \text{volume of the enzyme in ml}}$$

Specific activity is expressed as units per mg protein, where one unit is defined as one nmole of NADPH oxidized per min.

Preparation of affinity matrix

Affinity matrix was prepared by coupling glutathione (GSH) to epoxy activated sepharose 6B as per the method of Simmons (1977). About 4g of epoxy-activated sepharose 6B was washed with 500ml of distilled water followed by 40ml of 50mM phosphate buffer pH 7.0. Matrix was transferred to another flask and the volume was adjusted to 20ml with the same buffer and N_2 gas was passed through for 5 min. To this 4ml of GSH (400mg of GSH in 4ml of distilled water, pH adjusted to 7.0 with KOH) was added and allowed to stay for 24hr at 37°C with constant stirring. The coupled gel was washed with 100ml of distilled water followed by 100 ml of 0.5 M KCl in 0.1M sodium acetate pH 4.0 and 0.5M KCl in 0.1M sodium borate buffer pH. 8.0. Finally the matrix was transferred to 10mM potassium phosphate buffer pH. 7.0 containing 150mM KCl. This matrix

was packed into a Biorad column and used for purification of GSTs.

Purification of GSTs

Tissue homogenate (10%) supernatant (10,000 rpm for 45 minutes) was prepared and centrifuged at 1,05,000 X g for 1 hour. The supernatant obtained is referred to as the cytosolic fraction. Cytosolic fraction after dialysis was loaded onto the GSH-Sepharose 6B affinity column (Simmons, 1977) previously equilibrated with 10mM potassium phosphate buffer pH 7.0 containing 0.15M KCl and then washed with the same buffer till the protein content dropped to zero (by spectrophotometric detection). The affinity bound GSTs were eluted with 10mM potassium phosphate buffer pH 7.0 containing 10mM GSH and 1ml fractions were collected. Active fractions were pooled and concentrated by lyophilization.

Antisera Production

Purified protein from affinity column were used to raise the antibodies. Rabbits (New Zealand white male, 2 months old), prior to the injection of the sample, were bled and stored as pre-immune sera. The samples were injected subcutaneously, which were emulsified in Freund's complete adjuvant in 1:1 ratio. The booster injections were given with the sample in incomplete Freund's adjuvant and continued for 3 months giving booster every 15 days. Rabbits were bled a week after the final booster, serum aliquots were further subjected to purification using DEAE-Cellulose column and stored at –20°C as primary antibody.

Antibody purification

Antibodies were purified by addition of equal volume of saturated ammonium sulfate to the serum at room temperature for 30 min. IgG pellet was collected by centrifugation at 8000g for 15 min. The white IgG pellet dissolved in a small volume of 70mM sodium phosphate (pH: 6.3) and dialyzed overnight against the same buffer (3 changes). The antibodies were further purified by negative absorption by DE-52 cellulose column (5ml-bed volume) with 70mM sodium phosphate (pH 6.3). The flow through fractions showing a high absorption at 280nm were pooled and precipitated by adding equal volumes of saturated ammonium sulfate solution. The IgG pellet obtained was dissolved in small volume of PBS and dialyzed against the same buffer. This was aliquoted into 1ml fractions and stored at –20°C. The purity was checked on SDS-PAGE.

Protein determination

Protein content in the chromatographic fractions was determined spectrophotometrically by the procedure of Warburg and Christian (1941) by measuring the absorbance at 280nm. Protein content in the samples like crude homogenate and cytosolic fraction was measured by the method of Bradford (Bradford, 1976).

SDS-PAGE

Vertical slab gel electrophoresis was performed according to the method of Laemmli (1970). The separated proteins were stained either with coomassie blue or silver staining.

Silver staining

Gels were stained with silver nitrate by the method of Blum (Blum, 1981). Proteins in gels were fixed with a solution of 50% methanol, 7.5% acetic acid and formaldehyde (50µl/100ml) for 1h. Gels were treated thrice with 50% ethanol for 30 min. each. The gels were treated with 2mg/100ml sodium thiosulfate for 1 min and were washed thoroughly with distilled water. The gels were treated with 0.2% silver nitrate solution containing formaldehyde (50µl/100ml) for 30 min to 1h, rinsed with distilled water and color was developed with 3% sodium carbonate solution containing formaldehyde (500 µl/1000ml). Color development was stopped by 1% acetic acid and gels were preserved in 5% acetic acid.

Western blotting

Proteins resolved on SDS-PAGE were electro blotted onto nitrocellulose membranes by wet transfer in 25mM Tris-HCl (pH 8.3) buffer containing 192mM glycine and 20% methanol (Towbin, 1979). The membrane was incubated overnight in blocking solution (5% dried minimal fat milk and 0.25% Tween 20 in TBS) at 4^0C and probed with primary antibody in 5% fat free milk, followed by secondary antibody (1:1000 goat anti-rabbit IgG linked to alkaline phosphatase

(1mg/ml, B.Genei, India) in 5% milk and colour was developed in ALP buffer (100mM Tris and 5mM $MgCl_2$; pH9.2) containing 0.033% nitro blue tetrazolium (NBT) and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The colour reaction was stopped with distilled water.

MALDI analysis

In-gel protein samples were digested with trypsin for four and half hours at 40°C. The resulting peptides were cleaned using zip-tip. 1µl aliquot was spotted onto the sample plate with 1µl of matrix (A Cyano-4 hydroxy Cinnamic Acid , 10mg/ml in 70% v/v acetonitrile and 1% trifluoro acetic acid and allowed to air dry. This sample was further analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometer with a micromass TOF (time of flight) spec. A nitrogen laser of 337nm was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range of 500Da to 3500Da.

DNA isolation

The blood samples and the breast cancerous and normal tissue samples were used to isolate the DNA.

DNA isolation from blood

5ml of the blood sample was used to isolate the DNA. To the 5ml of the blood 4 volumes of solution A (10mM Tris pH 8.0, 5mM $MgCl_2$ 320mM sucrose, 1% Triton X-100) was added, mixed well and centrifuged at 2,500 rpm for 5 min. The supernatant was decanted. To the pellet 2ml of solution B (400mM Tris pH

8.0, 60mM EDTA, 150mM NaCl, 10% SDS) was added, mixed well and 750µl of solution C (5M sodium perchlorate) was added. It was mixed thoroughly with 2ml of Tris saturated phenol and 2ml of chloroform:isoamyl alcohol in 24:1 ratio was added and centrifuged at 3000 rpm for 10min. To the supernatant equal volume of chloroform:isoamylalcohol (24:1) was added and centrifuged at 3000 rpm for 10min. Double the volume of absolute alcohol was added to the supernatant to precipitate the DNA. The DNA was spooled and washed with 70% alcohol. The pellet was dried and dissolved in 100µl of Tris EDTA (TE) pH 8.0.

DNA isolation from tissue

200mg tissue was homogenized in eppendorf tubes with 200µl of buffer A (40mM Tris pH 8.0, 5mM NaCl and 1mM EDTA pH 8.0), 300µg of proteinase K and a final concentration of 2% SDS were added and incubated at 37°C for over night. 200µl of buffer B (4mM Tris pH 8.0, 1.5mM NaCl and 1.2mM EDTA) was added and mixed. To this 100µl of buffer C (5M sodium perchlorate) was added and mixed well. Equal volume of phenol and chloroform:isoamylalcohol (24:1) was added and centrifuged at 6000 rpm for 5min. To the supernatant equal volume of chloroform:isoamylalcohol was added and centrifuged at 6000rpm for 5min. Double the volume of absolute alcohol was added to the supernatant to precipitate the DNA and centrifuged at 10,000rpm for 15min. The pellet was washed with 70% alcohol, dried and dissolved in 200µl of TE.

RNA isolation

The RNA was isolated using Trizol reagent and the isolation steps were followed as mentioned in the instructions booklet provided by Invitrogen. 100mg of the tissue was homogenized in 1ml of Trizol reagent, incubated at 10-30°C for 5 min. This was centrifuged at 7,500 X g for 10 min and the supernatant was collected. 0.2ml of chloroform was added and tubes were vigorously mixed for 15 Sec and incubated at 30°C for 2-3min. This was centrifuged at 12,000 X g for 10 min. at 4°C. The supernatant was collected and equal volume of isopropyl alcohol was added. The samples were incubated at 10-30°C for 10min and centrifuged at 12,000 X g for 10 min at 4°C. The RNA pellet was collected and washed with 75% ethanol. The pellet was dried and dissolved in millipore water/molecular grade formamide. RNA was electrophoressed on a formaldehyde/MOPS/EDTA agarose gel (1.2%).

PCR analysis

The template DNA used for PCR analysis was from the breast cancerous and normal tissue samples or the DNA of blood samples collected from normal and those suffering from breast cancer.

The PCR mixture contained

10x PCR buffer - 2µl

100x molecular grade BSA - 2µl

2mM dNTPs - 2µl

Forward primer - 5pmoles

Reverse primers - 5pmoles

Taq polymerase - 1unit

Template DNA - 50ng

The total reaction volume was made upto 20µl using autoclaved double distilled water. The PCR primers used were specific to the genes of GSTM1, GSTT1, and GSTP1. The following are the sequences of the specific primers used.

GSTM1

5'-GAACTCCCTGAAAAGCTAAAGC-3' – FORWARD

5'-GTTGGGCTCAAATATACGGTGG-3' – REVERSE

These primers would amplify a product of 215bp,

GSTT1

5'-TTCCTTACTGGTCCTCACATCTC-3' - FORWARD

5'-TCA CCGGATCATGGCCAGCA-3' – REVERSE

These primers would amplify a product of 480bp

GSTP1

5'- GTAGTTTGCCCAAGGTCAAG -3' - FORWARD

5'- AGCCACCTGAGGGGTAAG -3' – REVERSE

These primers would amplify a product of 432bp

SSCP analysis

The PCR gene product of specific gene was used for this analysis. 3µl of the product was mixed with 3µl of SSCP gel loading dye (950µl of formamide,

1µl of 10N NaOH, 40µl of 0.5M EDTA, 9µl of sterile water, 0.005g of bromophenol blue, 0.005g of xylene cyanol). This sample was denatured at 96°C for 10 min. This sample is immediately kept on ice for 10min. The denatured DNA would remain single stranded and make its own three dimensional conformations. These samples are electrophoressed on 7% poly acrylamide gel at a constant voltage at 4°C. Then the gel was stained either with ethidium bromide or silver nitrate to monitor the DNA bands

RT-PCR analysis

The RNA isolated was subjected to reverse transcriptase PCR analysis using primers of different classes of GSTs. The reaction mixture contained

10x RT-PCR buffer - 2µl

Forward primer - 5pmoles

Reverse primer - 5pmoles

$MgCl_2$ – 0.6µl

Taq polymerase - 1unit

Reverse transcriptase enzyme - 2 units

Template RNA - 200ng

The total reaction volume was made to 20µl using double distilled water. The specific primers to GSTM1, GSTT1, and GSTP1 (sequence as mentioned above) genes were used to amplify the respective products. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers were used to amplify the product as an internal standard.

CHAPTER I

Title: Antioxidant defenses in breast cancers

Introduction

Glutathione is a tripeptide (γ- glutamyl cysteinyl glycine) molecule playing a vital role in many cellular functions, especially as an antioxidant molecule during oxidative stress. It acts as a substrate/co-substrate for the enzymes like glutathione S-transferases, glutathione peroxidases and glutathione reductase.

The enzyme glutathione reductase (GR) participates in the formation of disulfide bonds of many proteins and also helps in the regeneration of reduced glutathione from oxidized glutathione which thus participate in the metabolism of xenobiotics (Arrick, 1984). Glutathione S-transferases (GSTs) are a major group of phase-II drug detoxification enzymes which play a pivotal role in the detoxification of an array of xenobiotics. GSTs are multifunctional, multigene, dimeric proteins. These enzymes detoxify the compounds that are hydrophobic or electrophilic in nature either endogenous or exogenous in origin. The GSTs detoxify these compounds by conjugating with the reduced glutathione and thereby making them hydrophilic and more easily extractable compounds (Mantle, 1990; Mannervik, 1992)

Apart from detoxification, GSTs catalyze several other reactions. The second major function attributed to GSTs is the peroxidase activity denoted as non-selenium glutathione peroxidase activity (Non-Se GSH Px) which reduces many harmful peroxides in the cell. In addition to non-se GSH Px activity of GSTs, there are other peroxidases including glutathione peroxidases (GPx).

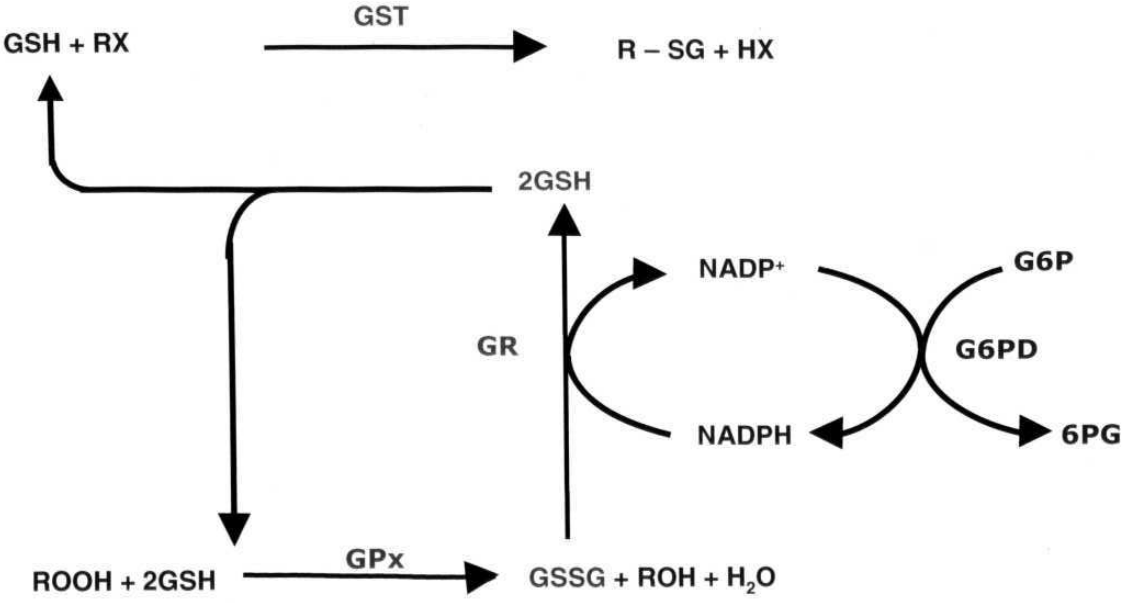
Glutathione peroxidases are Selenium dependent enzymes that play a major role in maintaining the redox status of the cell by reducing peroxides generated within the cell and by coordinating with other antioxidant enzymes during severe oxidative stress (Cheng, 1999). A schematic presentation of glutathione metabolizing enzymes is shown in figure 1.1.

Oxidative stress arises when there is an imbalance between the oxygen free radicals (OFRs) production and their scavenging antioxidants. Excess generation of OFRs can cause oxidative damage to biomolecules resulting in lipid peroxidation, mutagenesis and carcinogenesis.

Carcinoma of the breast is the third most common in the world and second most common cancer causing highest morbidity and mortality in Southern India (Gajalaxmi, 1997). The etiology of breast cancer is multifactorial. The hormonal, genetic and environmental factors appear to interplay in the pathogenesis of breast cancer (Russo, 2000). Increased or continuous exposure of cells to endogenous hormones is recognized as a major risk factor in the development of breast cancer. (Clemons, 2001). OFR-induced lipid peroxidation has been implicated in malignant transformation (Scot, 1991). The present work has been designed to evaluate total glutathione and the enzyme activities of GST, GR, GPx and peroxidase activity of GSTs in the breast cancer and adjoining normal tissues.

Environmental estrogens are naturally occurring (Eg. Phytoesrogens in plants) or synthetic chemicals that can act like human estrogen made by the

Fig. 1. 1. Glutathione metabolism



ovary. The greatest concern is over synthetic xenoestrogens that are not easily broken down, and that can accumulate and be stored in the body's fat cells, including breast fat. Xenoestrogens can mimic the effect of human estrogen because they have a chemical structure that allows them to fit into the estrogen receptor the way a key fits into a lock. Some xenoestrogens increase cell division and thus may contribute to breast cancer risk. Many different chemicals have been identified as being weak environmental estrogens. These include several pesticides (including some forms of DDT), the food preservatives BHT and BHA, the industrial detergent by-products nonyl- and octaphenol, compounds used in plastics including bisphenol A and some pthalates, the food dyes Red # 3, and solvent formaldehyde which was used in carpet manufacturing, and is still used in making plywood.

Many of these compounds are capable of binding to estrogen receptors and are hormonally active in animals causing either estrogenic or antiestrogenic effects. However, the observed effects are three orders of magnitude less than with naturally occurring estrogens. These hormonal properties are of interest since estrogens are growth factors for tissues of the breast and uterus. At lower doses, the xenoestrogens (aromatic organochlorines) may serve as tumor promoters to increase the rate by which a transformed cell grows into a clinically detectable tumor. Epidemiological studies present conflicting evidence regarding the association of xenoestrogens and breast and uterine cancers (Adami, 1995).

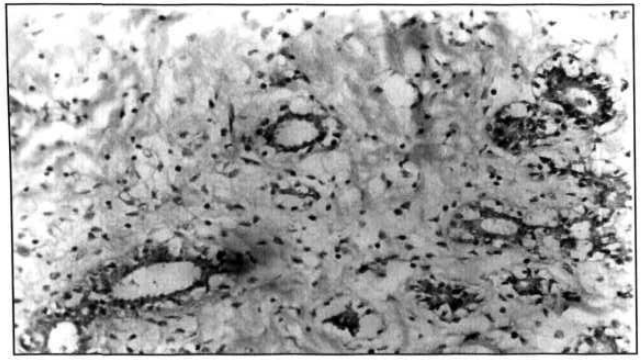
Results

Twenty four breast cancerous and corresponding normal tissue samples were collected from the patients coming to local hospitals. The histopathology was performed on the tissues to differentiate the cancerous tissue from the normal (Fig.1.2). The present work reports the variation in the concentration of glutathione and glutathione related enzymes like glutathione S-transferases (GSTs), glutathione reductase (GR), Se-dependent glutathione peroxidase (Se-GSH Px) and Non-Se-dependent glutathione peroxidase (Non-Se-GSH Px) in the breast cancer tissues in comparison to the corresponding normal tissues.

The concentration of reduced and oxidized glutathione in cancerous and normal tissues was measured according to the protocols mentioned in the methodology and the data presented in Table 1.1. The reduced glutathione levels were significantly higher (around two fold) in cancer tissues (Fig. 1.3, Table 1.1) compared to the corresponding normal tissues. The mean reduced glutathione levels in breast cancer tissues was 1.86mg/g tissue, with the values ranging from 0.072 to 6.56. The same in the corresponding normal tissues was 0.98mg/g tissue, with the values ranging from 0.146 to 2.386. The values in breast cancer tissues were significantly higher (52 %, $P< 0.01$) compared to the corresponding normal tissues.

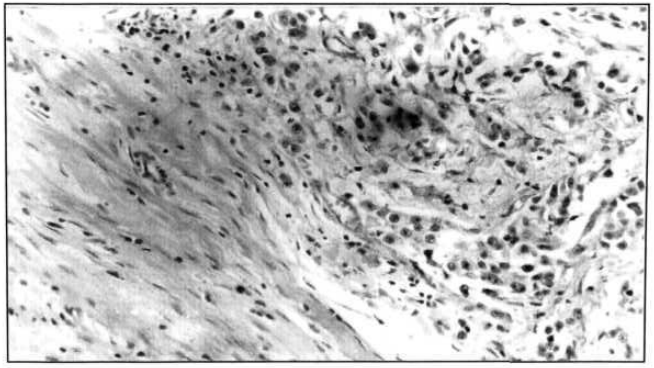
The mean of oxidized glutathione levels in breast cancer tissues was 9.85µg/g tissue, with the values ranging from 0.698 to 23.33. The same in the corresponding normal tissues was 2.89µg/g tissue, with the values from 1.0 to

Fig.1. 2. Histopathological sections of breast cancerous and normal tissues



Normal

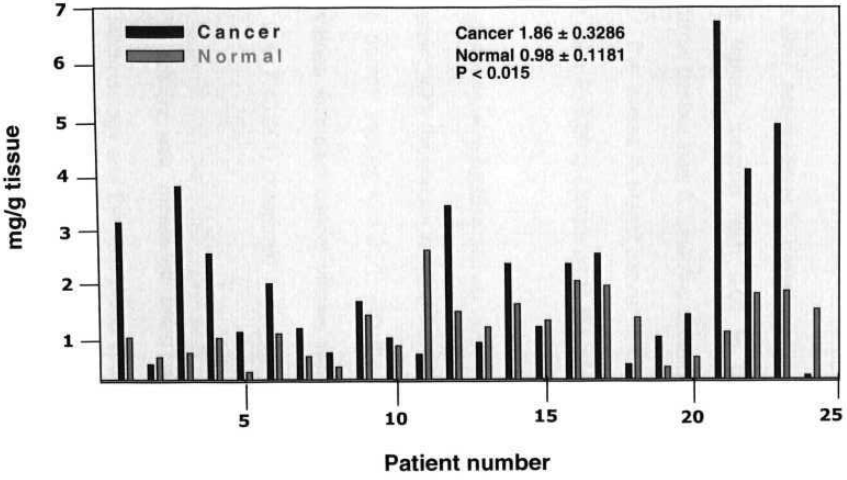
- *This section shows clear ducal and* stromal component, Regular sized nucleus, Not much mitosis seen



Cancer

- This section shows loss of ducal and stromal component, Irregular nuclear margins, spindle/irregular shaped nucleus and active atypical mitosis.

Fig.1. 3. Levels of reduced glutathione (GSH) in cancer and corresponding normal tissues



Reduced glutathione (GSH) was estimated in the breast cancerous and normal tissues. The average concentration of GSH in cancerous tissues was 1.86 mg/g tissue and that of normal was 0.98mg/g tissue. The concentration of GSH in cancerous tissue is significantly higher than that of normal tissues. The significance was calculated by Students t-test. The level of significance was set at $P<0.01$.

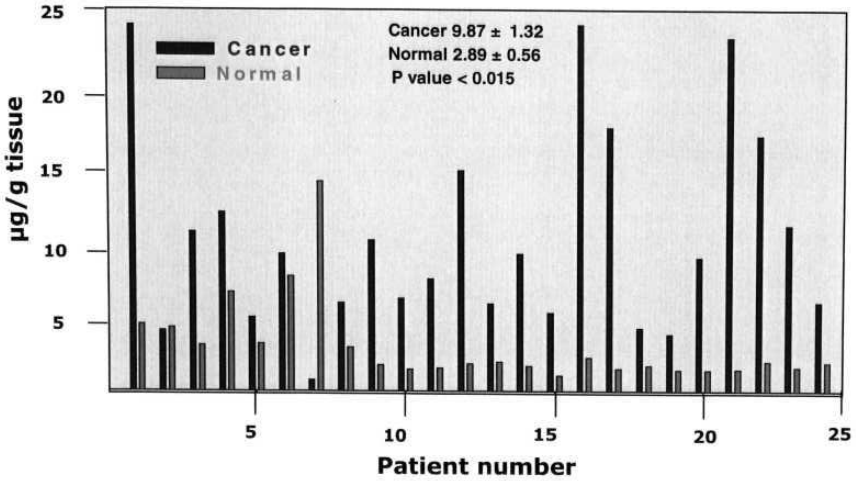
13.4 (Fig. 1.4). The values in breast cancer tissues were significantly higher (3.3 times higher, with $P<0.001$) compared to the corresponding normal tissues (Table 1.1). The levels of oxidized glutathione, in general, were much lower than reduced glutathione in the normal as well as cancerous tissues.

The mean GST activity level in breast cancer tissues was 0.36units/mg protein, with the values ranging from 0.01 to 0.87. The same in the corresponding normal tissues was 0.2units/mg protein, with the values from 0.01 to 0.75 (Fig. 1.5). The values in breast cancer tissues were significantly higher (1.8 times higher, with $P<0.001$) compared to the corresponding normal tissues (Table 1.2).

The mean glutathione reductase activity level in breast cancer tissues was 0.024units/mg protein, with the values ranging from 0.002 to 0.1. The same in the corresponding normal tissues was 0.01, with the values from 0.002 to 0.02 (Fig. 1.6). The values in breast cancer tissues were thus significantly higher (3.3times higher, with $P<0.001$) compared to the corresponding normal tissues (Table 1.2).

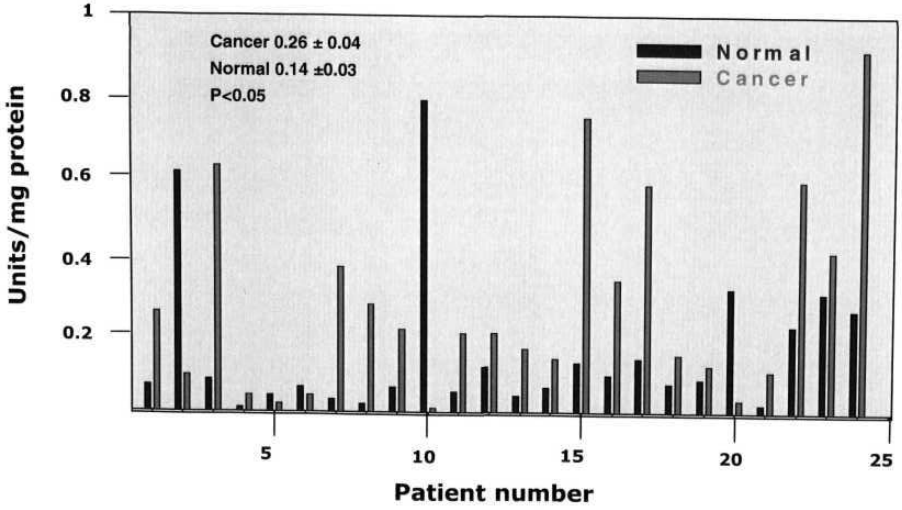
Se-GSH Px activity was measured using H_2O_2 as the substrate as mentioned in the methodology and the data presented in Table 1.3. As shown in the Table, Se-GSH Px activity levels in cancerous tissues were slightly higher (15.4%) compared to the normals (Fig. 1.7, Table 1.3) but the changes were non-significant.

Fig. 1. 4. Levels of oxidized glutathione (GSSG) in cancer and corresponding normal tissues



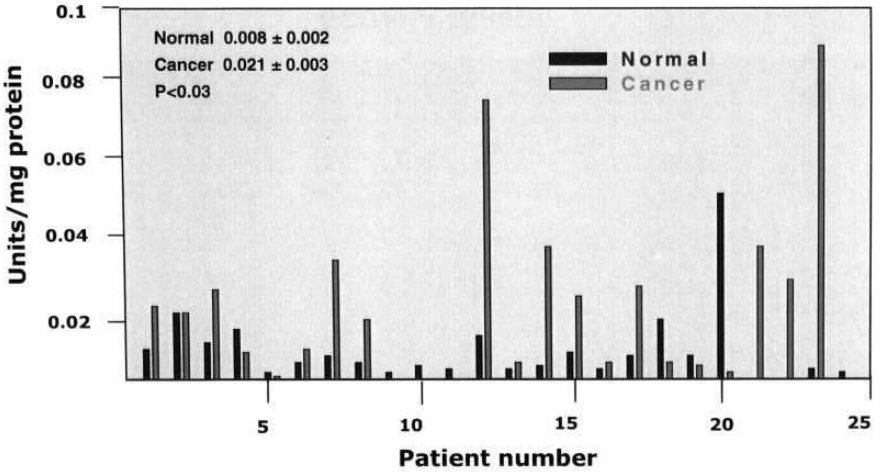
Oxidized glutathione (GSSG) was estimated in the breast cancerous and normal tissues and expressed in µg/g tissue. The average concentration of GSSG in cancerous tissues was 9.87 µg/g tissue and that of normal was 2.89 µg/g tissue. The concentration of GSSG in cancerous tissue is significantly higher than that of normal tissues. The significance was calculated by Students t-test. The level of significance was set at $P < 0.01$.

Fig. 1. 5. Total GST activity levels



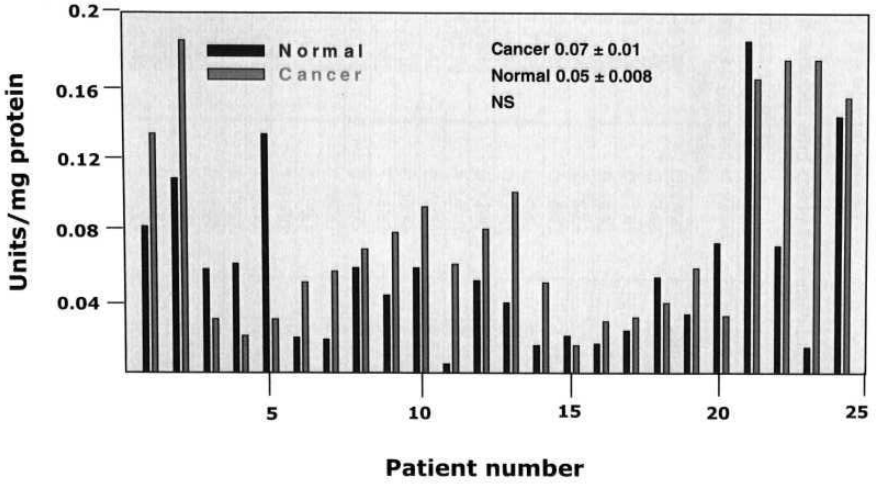
GST activity was estimated in human breast cancerous and normal tissues and values expressed in units/mg protein. The average activity of GSTs in cancerous tissues was 0.26units/mg protein and that of the normal tissues was 0.14units/mg protein. The activity of GSTs in cancerous tissues is significantly higher than that of normal tissues. The significance was calculated by students t-test. The level of significance was set at $P<0.05$. One unit of enzyme is defined as one µmole of thioether formed per minute.

Fig.1. 6. Glutathione reductase activity levels



GR activity was estimated in human breast cancerous and normal tissues and expressed in units/mg protein. The average activity of GR in cancerous tissues was 0.021units/mg protein and that of the normal tissues was 0.008units/mg protein. The activity of GR in cancerous tissues is significantly higher than that of normal tissues. The significance was calculated by students t-test. The level of significance was set at 0.03. One unit of enzyme is defined as one nmole of NADPH oxidized per minute.

Fig. 1. 7. Se-dependent glutathione peroxidase (Se-GSH Px) activity levels



Se-GPx activity was estimated in human breast cancerous and normal tissues and expressed in units/mg protein. The average activity of GR in cancerous tissues was 0.07units/mg protein and that of the normal tissues was 0.05units/mg protein. The activity of Se-GSH Px in cancerous tissues is higher than that of normal tissues but not significant. One unit of enzyme is defined as one nmole of NADPH oxidized per minute.

Non-Se-GSH Px activity was calculated as the difference between the total GSH Px activity levels and the Se-GSH Px activity levels (Fig. 1.8, Table 1.3). This showed no significant variation in the activity levels of cancerous tissues from those of the normal tissues.

Table 1.1. Levels of reduced and oxidized glutathione in normal and cancerous breast tissues of human subjects.

Patient number	Reduced glutathione (GSH) (mg/g tissue)		Oxidized glutathione (GSSG) (in µg/g tissue)	
	Cancer	Normal	Cancer	Normal
1	2.9000	0.7740	23.3300	4.2450
2	0.2900	0.4170	3.8530	4.0500
3	3.5700	0.4930	10.1840	2.9100
4	2.3300	0.7680	11.4320	6.3150
5	0.8800	0.1460	4.7070	3.0240
6	1.7700	0.8460	8.7840	7.3500
7	0.9420	0.4250	0.6980	13.4360
8	0.4910	0.2290	5.6340	2.7790
9	1.4370	1.1810	9.6660	1.6670
10	0.7640	0.6200	5.9200	1.3980
11	0.4610	2.3860	7.1930	1.4850
12	3.2050	1.2490	14.1500	1.7520
13	0.6790	0.9650	5.5920	1.8620
14	2.1330	1.3850	8.8100	1.6050
15	0.9660	1.0860	5.0100	1.0090
16	2.1360	1.8160	23.3900	2.1590
17	2.3190	1.7250	16.9200	1.4560
18	0.2740	1.1380	4.0500	1.6420
19	0.7810	0.2230	3.6450	1.3670
20	1.1940	0.3990	8.5840	1.3530
21	6.5600	0.8680	22.5840	1.4110
22	3.8740	1.5800	16.3880	1.9050
23	4.7020	1.6290	10.6560	1.5350
24	0.0720	1.2920	5.7090	1.8400
Average	**1.86**	**0.98**	**9.850**	**2.89**

Reduced glutathione

T value	2.5162402580
P value	0.0154136217
Degrees of Freedom	46

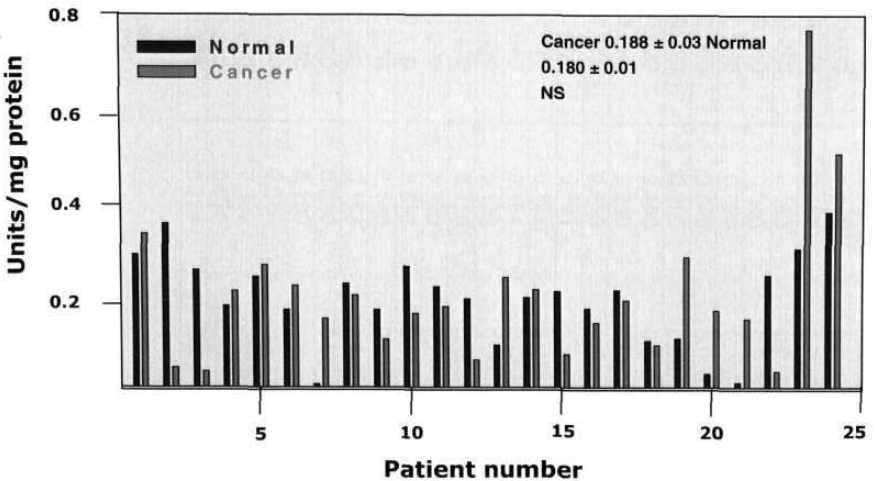
	C	N
Mean:	1.8637	0.9850
Std. Deviation:	1.6100	0.5787
Std Error:	0.3286	0.1181

Oxidized glutathione

T value	4.8579700877
P value	0.0000141353
Degrees of Freedom	46

	C	N
Mean:	9.8704	2.8981
Std. Deviation:	6.4701	2.7522
Std. Error:	1.3207	0.5618
95% Conf:	2.7321	1.1622

Fig. 1. 8. Non-Se-glutathione peroxidase (Non-Se GSH Px) activity levels



Non-Se-GSH Px activity was estimated in human breast cancerous and normal tissues and expressed in units/mg protein. The average activity of GR in cancerous tissues was 0.188units/mg protein and that of the normal tissues was 0.180units/mg protein. The activity of Non-Se-GSH Px in cancerous tissues is higher than that of normal tissues but not significant. One unit of enzyme is defined as one nmole of NADPH oxidized per minute.

Table 1.2. Activity levels of GSTs and GR in normal and cancerous breast tissues of human subjects

S.No	GST activity (Units/mg* protein)		GR activity (Units/mg** protein)	
	Normal	Cancerous	Normal	Cancerous
1	0.063	0.240	0.009	0.022
2	0.578	0.088	0.020	0.020
3	0.078	0.593	0.011	0.027
4	0.010	0.040	0.015	0.008
5	0.040	0.020	0.002	0.0008
6	0.060	0.040	0.005	0.009
7	0.030	0.350	0.007	0.036
8	0.020	0.260	0.005	0.018
9	0.060	0.200	-	-
10	0.750	0.010	-	-
11	0.050	0.191	-	-
12	0.110	0.192	0.013	0.084
13	0.040	0.154	0.003	0.005
14	0.060	0.130	0.004	0.040
15	0.120	0.710	0.008	0.025
16	0.090	0.320	0.003	0.005
17	0.130	0.550	0.007	0.028
18	0.070	0.140	0.018	0.005
19	0.080	0.113	0.007	0.004
20	0.300	0.030	0.056	0.002
21	0.020	0.100	-	-
22	0.210	0.560	-	-
23	0.290	0.390	0.003	0.100
24	0.250	0.870	-	-
Average	**0.206**	**0.364**	**0.01**	**0.0244**

GST

T value -1.9039872336

P value 0.0631780119

Degrees of Freedom 46

GR

T value -1.8970177612

P value 0.0663464001

Degrees of Freedom 34

	N	C
Mean	0.1462	0.2621
Standard deviation	0.1812	0.2369
Standard error	0.0370	0.0484
Mean	0.0109	0.0244
Standard deviation	0.0124	0.0275
Standard error	2.9316e-3	6.4781e-3

* one unit of enzyme is defined as µmoles of thioether formed/min.

** one unit of enzyme is defined as one n mole of NADPH oxidized per minute

Table 1.3. Activity levels of total GSH-Px, Se-GSH-Px and Non-Se-GSH-Px in normal and cancerous breast tissues of human subjects

S.No.	Total GSH-Px activity (Units/mg)[a]		Se-GSH-Px activity (Units/mg)[a]		Non-Se-GSH-Px activity (Units/mg)[a]	
	Normal	Cancer	Normal	Cancer	Normal	Cancer
1	0.350	0.443	0.080	0.130	0.270	0.313
2	0.450	0.220	0.106	0.180	0.334	0.040
3	0.296	0.062	0.057	0.030	0.239	0.032
4	0.226	0.217	0.060	0.021	0.166	0.196
5	0.356	0.280	0.130	0.030	0.226	0.250
6	0.178	0.258	0.020	0.050	0.158	0.208
7	0.025	0.196	0.019	0.056	0.006	0.140
8	0.270	0.187	0.058	0.068	0.212	0.188
9	0.201	0.175	0.043	0.077	0.158	0.098
10	0.306	0.242	0.058	0.091	0.248	0.151
11	0.212	0.255	0.006	0.06	0.206	0.165
12	0.232	0.135	0.051	0.079	0.181	0.056
13	0.125	0.324	0.039	0.099	0.086	0.225
14	0.200	0.250	0.016	0.050	0.184	0.200
15	0.218	0.084	0.021	0.016	0.196	0.068
16	0.178	0.161	0.017	0.029	0.161	0.132
17	0.223	0.210	0.024	0.0311	0.199	0.178
18	0.148	0.125	0.053	0.039	0.095	0.086
19	0.133	0.265	0.033	0.058	0.100	0.267
20	0.099	0.189	0.071	0.032	0.028	0.157
21	0.190	0.300	0.180	0.160	0.010	0.140
22	0.300	0.500	0.070	0.170	0.230	0.033
23	0.300	0.900	0.015	0.170	0.285	0.730
24	0.500	0.630	0.140	0.150	0.360	0.480
Average	**0.2382**	**0.275**	**0.056**	**0.078**	**0.181**	**0.1889**

			N	C
Total GSH Px				
T value	-0.8533420109	Mean	0.2382	0.2753
P value	0.3978914824	Standard deviation	0.1078	0.1842
Degrees of Freedom	46	Standard error	0.0220	0.0376
Se-GSH-Px				
T value	-1.5004391779	Mean	0.0570	0.0782
P value	0.1403337609	Standard deviation	0.0441	0.0533
Degrees of Freedom	46	Standard error	8.9943e-3	0.0109
Non-Se-GSH-Px				
T value	-0.2227331456	Mean	0.1808	0.1889
P value	0.8247292695	Standard deviation	0.0924	0.1529
Degrees of Freedom	46	Standard error	0.0189	0.0312

a. one unit is defined as one nmole of NADPH oxidized per minute

Discussion

Damage to the breast epithelium by oxygen free radicals (OFR) can lead to fibroblast proliferation, epithelial hyperplasia, cellular atypia and breast cancer. Overproduction of OFR coupled with antioxidant depletion is known to result in oxidative stress (Chattopadhyay, 2000; Matés, 1999). Glutathione, an important substrate for GSH Px, GR and GST has been documented to have regulatory effects on cell proliferation (Obrador, 1997). Increased level of GSH has been reported in both animal and human tumors (Skrydlewska, 2001; Balasenthil, 2000; Yong, 1997; Kumaraguruparan, 2002). GSH synthesis in tumor tissues was found to be associated with a high rate of cell proliferation (Estrela, 1997).

It is quite well known that reduced glutathione plays a central role in cellular defense against reactive oxygen species (ROS). In the ovarian tissues, the GSH concentration and GR activity were significantly higher in malignant tissues (Azza, 2000). Oxidative stress has long been related to carcinogenesis in human and animal cancers (Chen, 2000). ROS coupled with impaired antioxidant defenses can initiate lipid peroxidation and DNA damage leading to mutagenesis, carcinogenesis and cell death (Devi, 2000). Antioxidants by limiting the oxidative damage to DNA, protein and lipids reduce the risk of cancer (Sirens, 2001). Disturbed antioxidant defenses have been reported in patients with malignant lymphoma (Abou-Seif, 2000). Arivazhagan reported a decline in the antioxidants in erythrocytes in gastric cancer patients (Arivazhagan, 1997). Oxidant stress, on the other hand, was shown to cause upregulation of

antioxidant enzymes that render cells more resistant to subsequent oxidative insult (Halliwell, 2000). Thus there are conflicting reports in the literature, specifically with reference to glutathione status in cancer tissues. While reduced antioxidants may be responsible for neoplastic transformation, increased levels of GSH may be a compensatory mechanism to the induced cell proliferation in stabilized cancers. In the present study, both GSH and GSSG levels were significantly higher in breast cancer tissues, when compared to the corresponding normal tissues.

A significant increase in the activity of GSH Px, the first step of enzyme defense against H_2O_2 and other organic hydroperoxides has been reported in tumors (Doroshow, 1995; Iscan, 2002). Further more, GSH Px plays a key role in tumorigenesis by altering the lipoxygenase and cyclooxygenase pathways (Bryant, 1982; Capdevila, 1995; Reddanna, 1989). The higher activity of GSH Px in breast cancer cell lines was suggested to result from increased expression of GSH Px gene (Zachara, 1993). In the present study, however, no significant changes were observed in the activity levels of both Se-dependent and non-Se-dependent GSH Px activity levels. Glutathione S-transferases, another GSH dependent enzyme, is known to play a role in the detoxification mechanisms of a variety of xenobiotics (Azza, 2000). GSTs also play a role in the reduction of organic peroxides (Zhao, 1999). In the present study GST levels were significantly higher in breast cancer tissues, suggesting their possible induction. Similar induction of GSTs, especially the isozyme GSTP1-1, was reported in

various cancer tissues, cell lines and actively dividing cells (Saydam, 1997; Chen, 1997; Matsui, 2000; Kumaraguruparan, 2002). This induction of GSTs may probably be a compensatory mechanism in the actively proliferating cells for detoxification of various toxicants. It will be interesting to probe further and identify the specific GST isozymes induced in these breast cancer tissues.

The increased incidence of breast cancers in industrialized and urban centers, indicates that environmental pollutants/toxicants and stress factors play a major role in the etiology of breast cancers. The reactive oxygen species generated by environmental pollutants/toxicants and other stress factors might be responsible for neoplastic transformation in the breast tissues. Also some of the pollutants might be acting as xenoestrogens, specifically acting on breast tissues and initiating neoplastic transformation. Antioxidant metabolites and enzymes protect the tissues from ROS-mediated damage.

The present studies on breast cancer patients showed significantly higher levels of GSH and GSSG in cancer tissues when compared to the adjoining normal tissues. Associated with increased levels of GSH, GSTs were significantly higher in breast cancer tissues. Further in depth studies on GSH metabolism and GSTs isozymes would unravel their usefulness in the diagnosis/treatment of breast cancer.

The present work is undertaken to study the alterations in biomolecules which are highly sensitive to cellular stress/pathological conditions such as cancer when compared to the normal tissue. Antioxidant enzymes are one such

group of enzymes which get altered very quickly in pathological conditions like cancers.

CHAPTER II

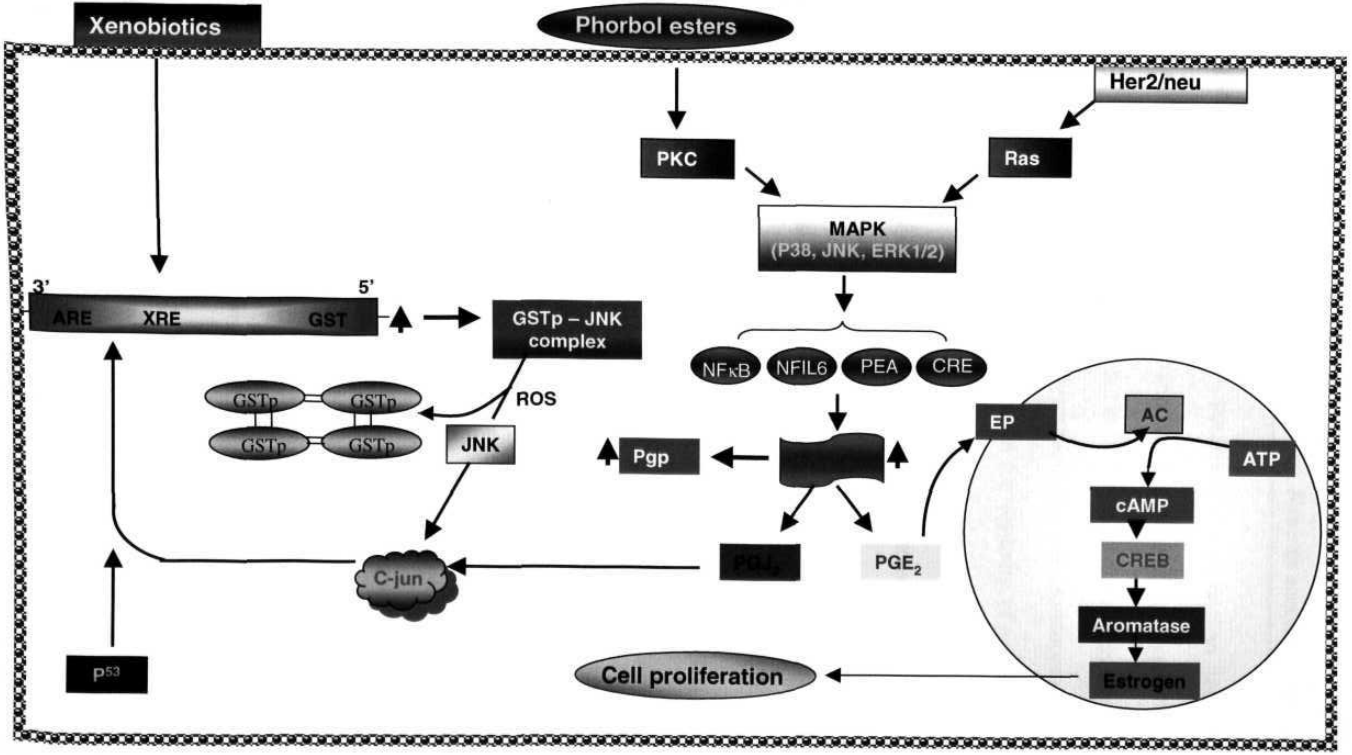
Title: Expression of selected markers of breast cancers

Introduction

A number of protein factor that are overexpressed in various cancers have become the selective markers. Among them Cyclooxygenase-2 (COX-2), phosphoprotein 53 (p^{53}), Apoptotic protease activating factor 1 (APAF1) and peroxisomal proliferator activated receptor (PPAR-γ) are important. COX-2, an immediate responsive gene that encodes a 71kDa protein which is up regulated at sites of inflammation (Scinicrope, 1993) and in some epithelial cancers, including human colon (Eberhart, 1994) gastric, (Ristimaki, 1997), esophageal (Zimmermann, 1999), and lung adenocarcinomas (Hida, 1998). Elevated levels of COX-2 mRNA and protein have been reported in breast cancer cell lines (Liu, 1996) but limited and conflicting data exist regarding the frequency of COX-2 expression in human breast cancers. Figure 2.1 depicts the various signal transductional pathways involved in the induction of COX-2 and the involvement of its products in initiating cell proliferation through activation of aromatase activity.

Prostaglandin (PGJ_2), a product of a COX, has been reported to induce the expression of GSTs (Kawamoto, 2000). PGJ_2 also forms a ligand for peroxisome proliferators-activated receptor–gamma (PPAR-γ). PPARs belong to the steroid/thyroid/retinoid receptor super family, and are nuclear lipid activable receptors that control a variety of genes in several pathways of lipid metabolism, including fatty acid transport, uptake, storage, intracellular binding, activation and

Fig. 2. 1. COX-2 and GST in cancer



catabolism (β-oxidation and ω-oxidation). PPARs are transcription factors activated by specific ligands and play an important role during cell signalling. Intensive study of PPARs is being carried out in both normal physiology and in the pathology of the lipid metabolism, inflammation and the development of atherosclerosis or diabetes. They also play a role in the regulation of growth and differentiation of cancer.

The p^{53} gene is localized on the short arm of chromosome 17 and it encodes 393 amino acid (53kDa) phosphoprotein, which is present at very low levels in normal cells. This molecule appears to play a major role in the maintenance of genomic integrity following DNA damage (Lane, 1992). p^{53} can either arrest the cells at the G1 phase of the cell cycle, thus providing time for the damage to be repaired (EL-Deiry, 1993; EL-Deiry, 1994; Nelson, 1994) or induces apoptosis (Lane, 1993). Both pathways prevent replication of damaged DNA and further accumulation of mutations. Cell containing biologically inactive p^{53} protein are devoid of such protective mechanism and they are genetically unstable. Genetic mutation is the most common pathway for p^{53} inactivation. Being mutated in approximately 50% of all tumors, p^{53} is currently considered the most frequently altered gene in human tumorigenesis.

Apoptotic protease activating factor 1 (APAF1) was originally isolated five years ago and shown to be the mammalian homologue of the C. elegans pro-apoptotic Ced 4 gene. Since then, the expression of APAF1 has been demonstrated to be involved in several cell death pathways, including the p^{53}

induced apoptosis and neuronal apoptosis (Hickman, 2002). APAF1 is a key regulator of stress and oncogene induced apoptosis mediated by the mitochondrial E2F transctiption factor. APAF1 is a transcriptional target for p^{53} in regulation of cell death (Fortin, 2001). Hence it is suggested that APAF1 could be a tumor suppressor. APAF1 is the molecular core of the apoptosome, a multiprotein complex mediating the so-called mitochondrial pathway of cell death. This protein gets released from mitochondrial membrane and forms apoptosome complex with cytochrome C released into the cytosol. This complex in turn activates caspases such as caspase 3 and provokes the apoptotic pathway.

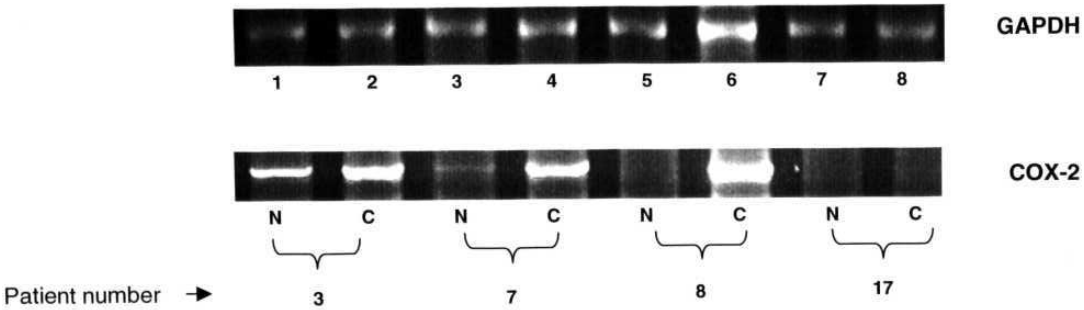
In view of the role played by COX-2, p^{53}, APAF1 and PPAR-γ in the pathogenesis of cancers, studies were undertaken on their expression at protein/mRNA level in breast cancer and the adjoining normal tissues.

Results

The proteins from cancerous and adjoining normal tissues of breast cancer patients were isolated, separated on SDS-PAGE, transferred onto nitrocellulose membrane and then probed with respective antibodies. In the present study the expression of COX-2, p^{53}, PPAR-γ and APAF-1 was analyzed at protein/mRNA level in breast cancer and the adjoining normal tissues.

Cyclooxygenase-2 expression was studied at mRNA level by employing RT-PCR method. The average expression of all the samples studied showed an overexpression of COX-2 in cancer tissues (Fig. 2. 2). In normal tissues basal level of expression of COX-2 was observed. Lane 1 shows the expression of

Fig. 2. 2. COX-2 expression in breast cancer and normal tissues

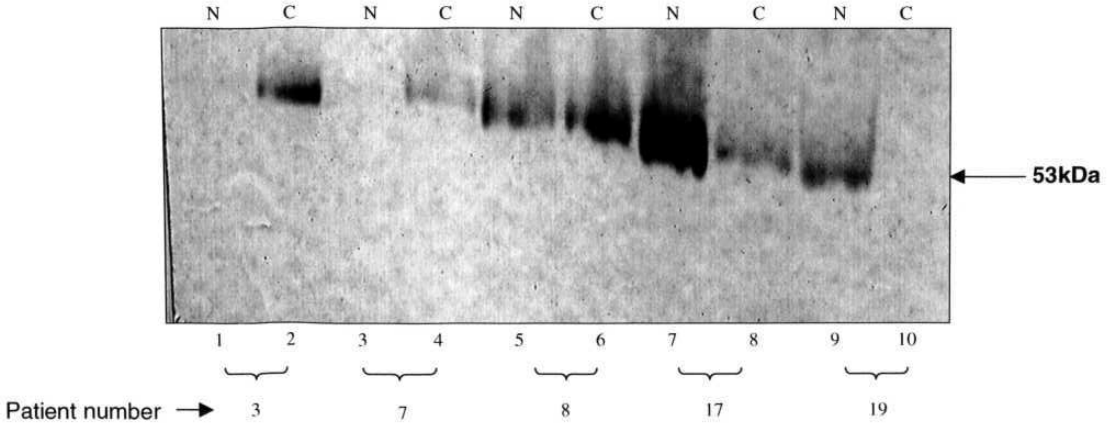


RT-PCR analysis of COX-2 gene of human breast normal and cancer tissues. The RT-PCR products were separated on 1% agarose gel electrophoresis showing over expression of COX-2 in both normal and cancer tissues. Lane 1 and 2 are the RT-PCR products from normal and cancer tissues respectively of patient number 3. Lane 3 and 4 are the RT-PCR products from normal and cancer tissues respectively of patient number 7. Lane 5 and 6 are the RT-PCR products from normal and cancer tissues respectively of patient number 8. Lane 7 and 8 are the RT-PCR products from normal and cancer tissues respectively of patient number 17. Glyceraldehyde phosphate dehydrogenase gene primers were used as internal control.

COX-2 in normal tissue which is relatively less compared to the corresponding cancerous tissue shown in lane 2. Similar overexpression of COX-2 was observed in cancer tissues of patients # 7 and 8 (lanes 3 to 6). In patient # 17, COX-2 mRNA is not detected both in normal and cancer tissues.

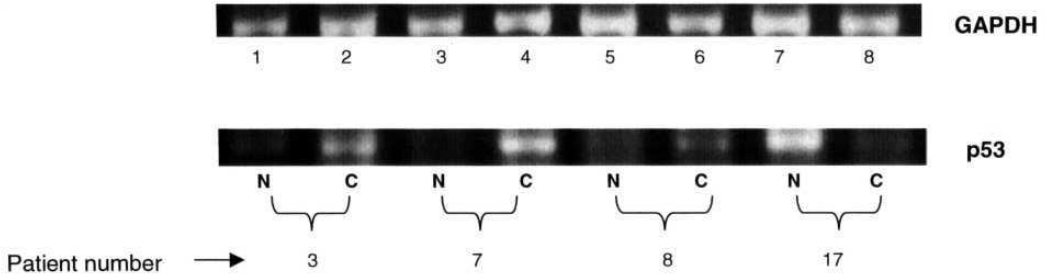
Western blot analysis of p^{53} is represented in figure 2. 3. In the samples of patients # 3, 7, and 8, higher levels of p^{53} protein was observed in cancer tissues (lane 2, 4 and 6) compared to that of the normal tissues (lane 1, 3 and 5). In patients # 17 and 18, however, the p^{53} protein levels in cancer tissues (lane 8 and 10) were much lower compared to that of normal tissues (lane 7 and 9). This was further studied at the mRNA level to have a better understanding of its expression. RT-PCR analysis of p^{53} gene in cancerous and normal tissues is represented in figure 2.4. The expression of p^{53} was higher in cancer tissues (lanes 2, 4 and 6) of patients # 3, 7 and 8 compared to the corresponding normal tissues (lane 1, 3 and 5). The expression of p^{53} in the cancer tissues of patients # 17 was less (lane 8) when compared to that in corresponding normal tissue (lane 7). This shows a correlation in the expression of p^{53} at mRNA level and protein level. The proteins of breast cancerous and normal tissues were separated on 10% SDS-PAGE and transferred onto the nitrocellulose membrane. These proteins were probed with PPAR-γ antibodies. The blot showed signal at a molecular weight of approximately 55kDa in the entire samples (Fig. 2.5). These studies on PPAR-γ showed no significant variation in its protein levels in cancerous tissues compared to the corresponding normal tissues. Similarly the

Fig. 2. 3. Western blot analysis of human breast cancer and normal tissue homogenate probed with p^{53} antibodies



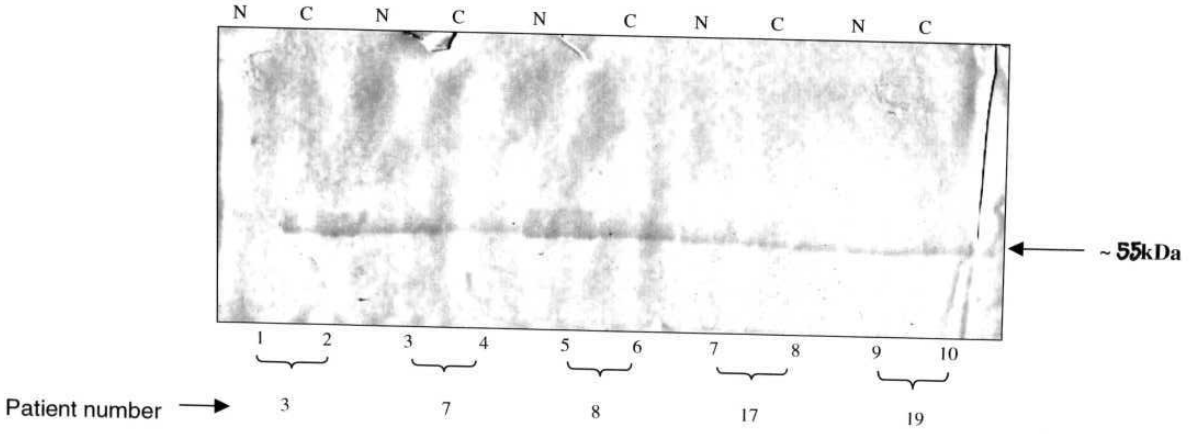
Proteins from breast cancerous and normal tissues were separated on 10% SDS-PAGE and blotted onto nitrocellulose membrane and probed with p^{53} antibodies. Lanes 1 and 2 are samples of normal and cancer tissue from patient number 3. Lane 3 and 4 are samples of normal and cancer tissues from patient number 7. Lane 5 and 6 are samples of normal and cancer tissue from patient number 8. Lane 7 and 8 are samples of normal and cancer tissues from patient number 17. Lane 9 and 10 are samples of normal and cancer tissues from patient number 19.

Fig. 2. 4. p^{53} expression in breast cancer and normal tissues



RT-PCR analysis of p53 gene of human breast normal and cancer tissues. The RT-PCR products from normal and cancer samples were separated on 1% agarose gel electrophoresis. Lane 1 and 2 are the RT-PCR products from normal and cancer tissues of patient number 3. Lane 3 and 4 are the RT-PCR products from normal and cancer tissues of patient number 7. Lane 5 and 6 are the RT-PCR products from normal and cancer tissues of patient number 8. Lane 7 and 8 are the RT-PCR products from normal and cancer tissues of patient number 17. Glyceraldehyde phosphate dehydrogenase gene primers were used as internal control.

Fig.2. 5. Western blot analysis of human breast cancer and normal tissue homogenate probed with PPAR-γ antibodies



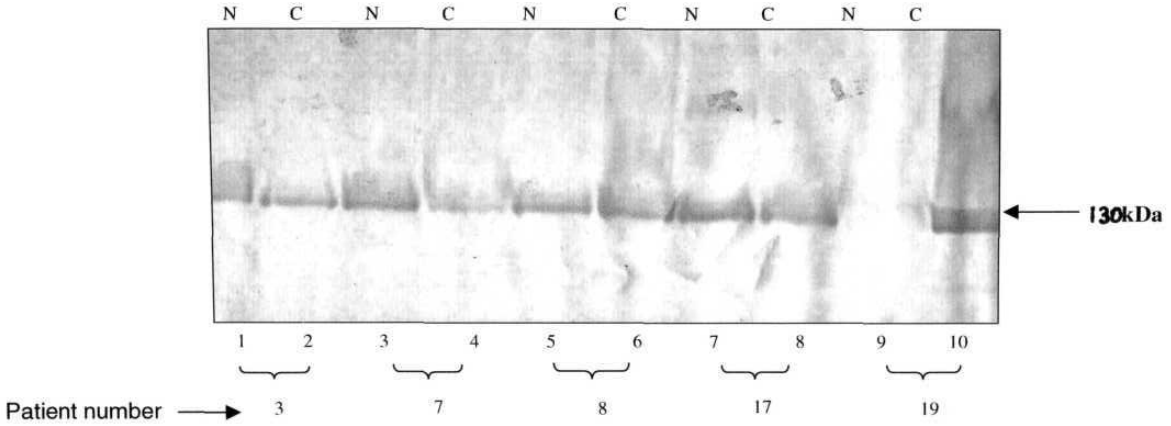
Proteins from breast cancerous and normal tissues were separated on 10% SDS-PAGE and blotted onto nitrocellulose membrane and probed with PPAR-γ antibodies. Lanes 1 and 2 are samples of normal and cancer tissue from patient number 3. Lane 3 and 4 are samples of normal and cancer tissues from patient number 7. Lane 5 and 6 are samples of normal and cancer tissue from patient number 8. Lane 7 and 8 are samples of normal and cancer tissues from patient number 17. Lane 9 and 10 are samples of normal and cancer tissues from patient number 19.

proteins were separated on 10% SDS-PAGE, transferred onto nitrocellulose membrane and probed with APAF1 antibodies. The Western blot showed a signal at a molecular weight of approximately 130kDa in all the samples (Fig. 2.6). The Western blot analysis showed no variation in the protein levels of APAF1 in cancer tissues compared to the corresponding normal tissues. In tissue sample of patient # 7 the levels of APAF1 were slightly lesser in cancer tissue compared to the normal tissue. In patient # 19, however, APAF-1 protein was overexpressed in cancer tissue when compared to that in normal tissue.

Discussion

Recent evidences indicate that COX-2 modulates the levels of multidrug resistance 1 (MDR-1, also known as p-glycoprotein) an efflux pump for chemotherapeutic agents. This raises the possibility that elevated levels of COX-2 contribute to multidrug resistance in tumors (Patel, 2002). Overexpression of COX-2 in neoplasia of breast cells leads to increased production of PGE_2, which, in turn, stimulates the expression of CYP19 (Aromatase) in stromal cells (Zhao, 1996). Consequently estrogen biosynthesis is enhanced, which leads to increased growth of neoplastic epithelial cells (Patel, 2002). The present study showed the overexpression of COX-2 in breast cancer tissues compared to the corresponding normal tissues of most of the patients. Similar overexpression of COX-2 in breast cancers was reported with no detectable expression in normal breast tissue (Parret, 1997). Another recent study has demonstrated COX-2 overexpression in various human malignancies, especially in breast cancer

Fig.2. 6. Western blot analysis of human breast cancer and normal tissue homogenate probed with APAF-1 antibodies



Proteins from breast cancerous and normal tissues were separated on 10% SDS-PAGE and blotted onto nitrocellulose membrane and probed with APAF 1 antibodies. Lanes 1 and 2 are samples of normal and cancer tissue from patient number 3. Lane 3 and 4 are samples of normal and cancer tissues from patient number 7. Lane 5 and 6 are samples of normal and cancer tissue from patient number 8. Lane 7 and 8 are samples of normal and cancer tissues from patient number 17. Lane 9 and 10 are samples of normal and cancer tissues from patient number 19.

(Spizzo, 2003). Studies from mouse models of mammary tumorigenesis and from human breast cancer cell lines provide evidence that COX-2 overexpression plays an important role in the pathogenesis of malignant breast cancer in humans. (Singh, 2002).

COX-2 mRNA is shown to be overexpressed in both human breast cancer and adjacent non cancer tissue (ANCT), suggesting that paracrine effects may be important in the carcinogenesis. It was also shown that overexpression of COX-2 is associated with the overexpression of vascular epidermal growth factor – 189 (VEGF-189) and therefore tumor angiogenesis (Kirkpatrick, 2002). In the present study also, RT-PCR analysis showed the overexpression of COX-2 in most of the breast cancer tissues when compared to the corresponding normal tissues. This overexpression of COX-2 may possibly be involved in either promoting angiogenesis or in the development of multidrug resistance in patients undergoing chemotherapy. These studies suggest the possible use of COX-2 specific inhibitors in the treatment of breast cancers also, in addition to their reported chemotherapeutic role on other cancers (Thimothy, 2002; Nagatsuka, 2002)

Though mutations in p^{53} genes were not analyzed in the present study, it is known that p^{53} gene gets mutated in most of the cancers including breast cancers (Bishop, 1996; Greenblatt, 1994; Ziyaie, 2000). The overexpression of such mutated p^{53} protein would be less effective in suppressing the tumor promotion. The present study revealed an overexpression of p53 in most of the

breast tumors compared to the corresponding normal tissues. Further studies, however, are required to find out the mutations in the p^{53} gene in breast cancer tissues. Over all, it appears that p^{53} overexpression and p^{53} mutations are rare events in normal breast and in benign lesions. However p^{53} abnormalities may occur relatively frequently in ductal carcinoma insitu (DCIS), being more common in high grade or comedo-type DCIS (i.e. those lesions that histologically have highest risk of progressing to invasive cancers. (Bartck, 1990; Eriksson, 1994; Poller, 1993; O'Malley, 1994).

p^{53} makes APAF1 as a key transcriptional target in regulating cell death (Fortin, 2001). The study on APAF1 through Western blot analysis has revealed no alteration in breast cancerous tissues as compared to the normal counter parts. p^{53}, though overexpressed in cancer tissues is inefficient to modulate the expression of APAF1. This suggests that the normal function of p^{53} has altered probably due to gene mutations and hence not able to affect the expression of APAF1 during cellular stress conditions such as cancer.

Similarly the other important molecular marker protein which has drawn much attention recently is PPAR-γ. A certain number of analyses suggest a role of PPAR-γ in cell growth arrest (Desvergne, 1999). In that respect, the physiological model of adipocyte conversion provides a valuable tool to study cell cycle arrest and terminal differentiation. Study based on malignant cells clearly support the concept of PPAR-γ being implicated in cell cycle withdrawal. Primary human liposarcoma cells, which express high levels of PPAR-γ can be

stimulated to undergo terminal differentiation by treatment with PPAR-γ ligands (Tontonoz, 1997). Activation of PPAR-γ also induces reduction in growth rate and clonogenic capacity of human breast cancer cells in culture. Hence PPAR – γ activators are efficient anti proliferators (Kubota, 1998). Also PPAR-γ was shown to be involved in transctiptional down-regulation of aromatase, a key enzyme in estrogen biosynthesis (Memisoglu, 2000). In the present study the expression of PPAR-γ showed no alteration in cancerous tissues compared to the corresponding normal tissues. This suggests that there is no anti proliferative effect of PPAR-γ in breast cancer tissues as a result of its unaltered expression.

Local estrogen biosynthesis in breast adipose tissue catalyzed by CYP19 (Aromtase), contributes to the growth of breast carcinomas. Aromatase expression is regulated by a number of alternative promoters in normal adipose tissue. However, in breast adipose containing a tumor, aromatase expression is regulated by proximal promoter II in response to tumor derived factors. Previously it was shown that PPAR-γ ligands inhibit aromatase expression in normal breast tissue mediated by promoter I.4. PPAR-γ mediated inhibition of aromatase expression occurs through an indirect mechanism of action. Because ligands for PPAR-γ inhibit aromatase expression in healthy breast adipose tissues such compounds could find utility in the treatment of estrogen-dependent breast cancers (Rubin 2002).

COX-2 and PPAR-γ may contribute to breast cancer induction either directly or via their effects on factors known to influence tumor development Eg:

nuclear factor-kappa B and VEGF (Bawadi, 2002). Inhibition of COX-2 or activation of PPAR-γ prevents mammary carcinomas in experimental animals with little toxicity (Badawi, 2002). Combinational treatment with COX-2 inhibitor and PPAR-γ agonists may produce synergistic anti-tumorigenic effects without significant toxicity and therefore, be an effective strategy to prevent human breast cancers (Ehrmann, 2002).

In conclusion overexpression of COX-2 and p^{53}, along with unaltered levels of PPAR-γ, and APAF1 was observed in the present study on breast cancer tissues. These observations suggest the mechanisms operating in the cancer cell are in favour of cell proliferation rather than cell cycle arrest or progression of apoptosis. Among the parameters studied in the present and previous chapters, highly significant elevation in GSTs was observed in breast cancer tissues. Since GSTs are multigene, multifunctional proteins involved in the detoxification of various xenobiotics, it will be interesting to probe further on the expression of individual subunits in breast cancer tissues and to analyze their correlation to the pathogenesis of breast cancers.

CHAPTER III

Title: Expression of GSTs in breast cancers

Introduction

In order to understand the role of GSTs in diseased conditions like cancers, it is important to isolate and identify the individual subunits. In the earlier chapter, it was shown that total activity of GSTs is higher in breast cancer tissues when compared to the corresponding normal tissues. However, the specific GST subunits induced in these cancerous tissues were not identified. Hence, the purification and characterization of GSTs and their expression at protein and mRNA level was undertaken in the present study. GSTs were purified by GSH affinity column chromatography from normal and cancerous tissues of human breast cancer patients. The level of expression of individual GSTs in normal and cancerous tissues was analyzed at protein (Western blot) and mRNA (RT-PCR) levels.

Results

Twenty four samples of breast cancer and their corresponding normal tissues were analyzed for the expression of GST subunits, at both protein and mRNA level.

Affinity column purification of GSTs:

The cytosolic fractions of normal and cancerous tissues, obtained as per the methods described in the methodology, were subjected to GSH affinity purification. Each fraction collected was studied for the enzyme activity and protein content. The typical GST elution profile from GSH affinity column is

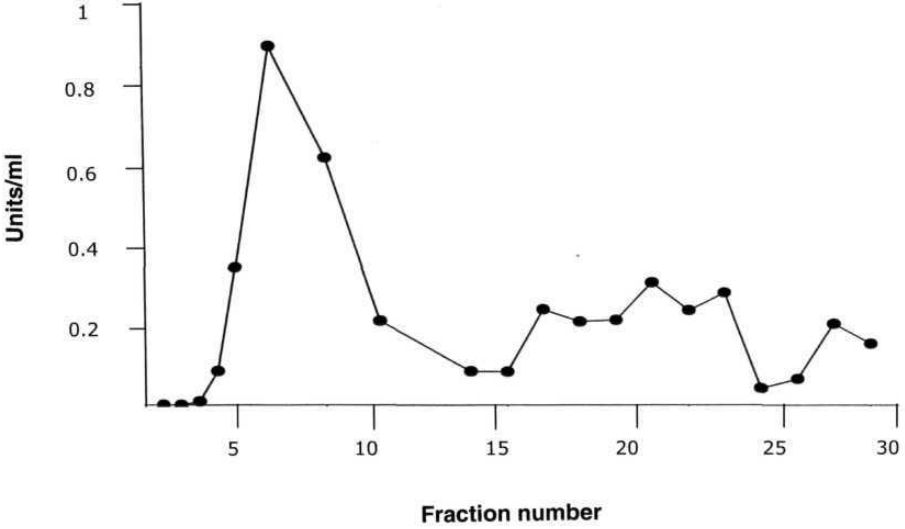
represented in figure 3.1 and the purification profile of cancerous and normal tissue GSTs is represented in Table 3.1 and Table 3.2 respectively. The fold purification obtained in cancerous tissues was 15 with specific activity of 21units/mg protein and an overall yield of 78%. In normal tissues the fold purification obtained was 12, the specific activity of 13 and the yield was 69%.

The purified GSTs from cancer and normal tissues were subjected to 12% SDS-PAGE analysis to check the purity and molecular weights (Fig. 3.2). 5µg of the purified GSTs were separated on SDS-PAGE. The GSTs from normal breast tissues were resolved into three bands (lane 3) and into four bands in cancerous tissues (lane 2) at a molecular weight range of 24 to 29kDa. These proteins were used for Western blot analysis using different class specific GST antibodies.

Western blot analysis:

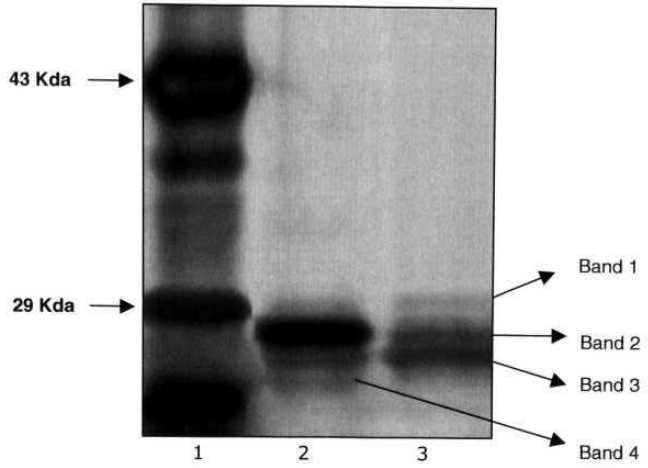
The Western blot analysis of GSTs with alpha class antibodies showed no change in the levels in cancerous tissues (lane 3) compared to the normal tissues (lane 2) (Fig. 3.3). The variation in the expression was estimated by scanning densitometry software which measures the band intensity. The scanning densitometric comparison of the band intensities between cancerous and normal tissues was shown as bar diagrams (Fig. 3.3). As shown in the figure, there is not much difference in the 26kDa protein bands of normal (lane 2) and cancer (lane 3) tissues. In the breast cancer tissues, however, an extra band of 70kDa protein was seen. No corresponding protein band was observed

Fig.3. 1. GSH affinity column elution pattern of Human breast cancerous tissue GSTs



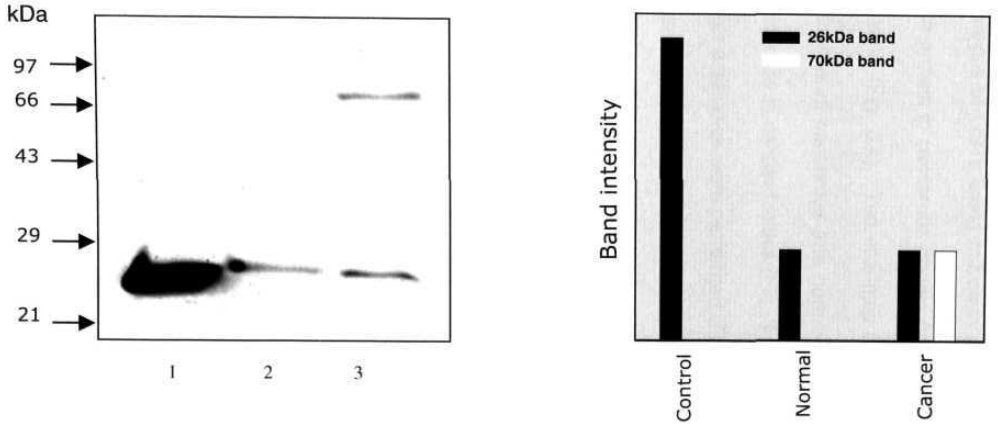
The elution profile of GSTs on GSH-affinity column chromatography from human breast cancerous tissue. The activity of each fraction was checked according to the method of Habig et al. and represented as units/ml

Fig.3. 2. SDS-PAGE analysis of GSTs purified from breast cancerous and normal tissues



GSH-affinity purified GSTs were separated on 12% SDS-PAGE from human breast cancerous and normal tissues and silver stained. Lane 1: Molecular weight markers, Lane 2: Affinity purified GSTs from human breast cancerous tissues, Lane 3: Affinity purified GSTs from human breast normal tissue GSTs

Fig. 3. 3. Western blot analysis of GSTs probed with GST alpha class antibodies



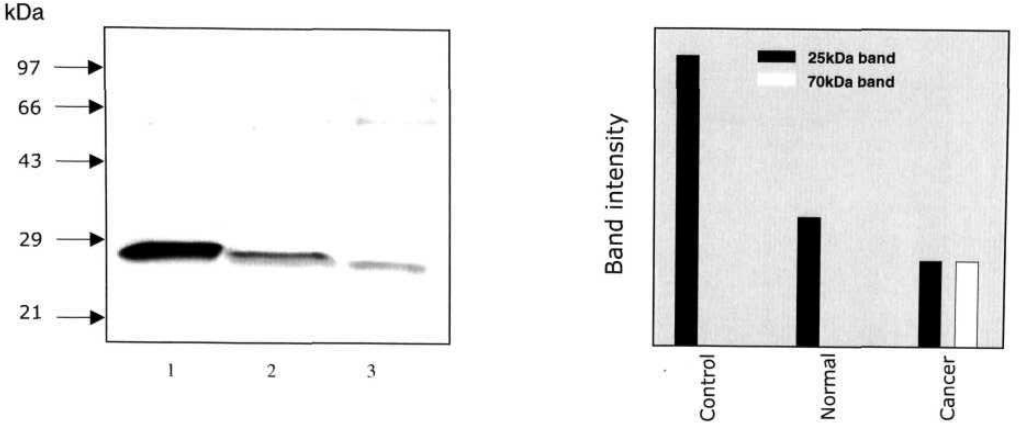
Affinity purified GSTS were from human breast cancer and normal tissues separated on 12% SDS-PAGE, transferred on to nitrocellulose membrane and probed with alpha class GST antibodies. Lane 1: human fetal liver GSTs, Lane 2: human breast normal tissue GSTs, Lane 3: human breast cancer tissue GSTs. The relative intensities of each band of 26kDa band are represented as solid bars and the 70kDa protein band as empty bars.

in the normal tissues.

Western blot analysis of GSTs using mu class antibodies showed minor variation in the levels of protein in cancerous tissues (lane 3) when compared to the normal tissues (lane 2) (Fig. 3.4). The scanning densitometric comparison between the band intensities of cancerous and normal tissues showed a slight decrease in the band intensity in cancer tissues (Fig. 3.4). Similarly the Western blot analysis with pi class antibodies were performed (Fig. 3.5). As shown in the figure, no signal was detected in normal tissue (lane 2). In cancerous tissues (lane 3), however, two protein bands were prominently seen, one in the range of 24kDa and the other at 70kDa (lane 3).

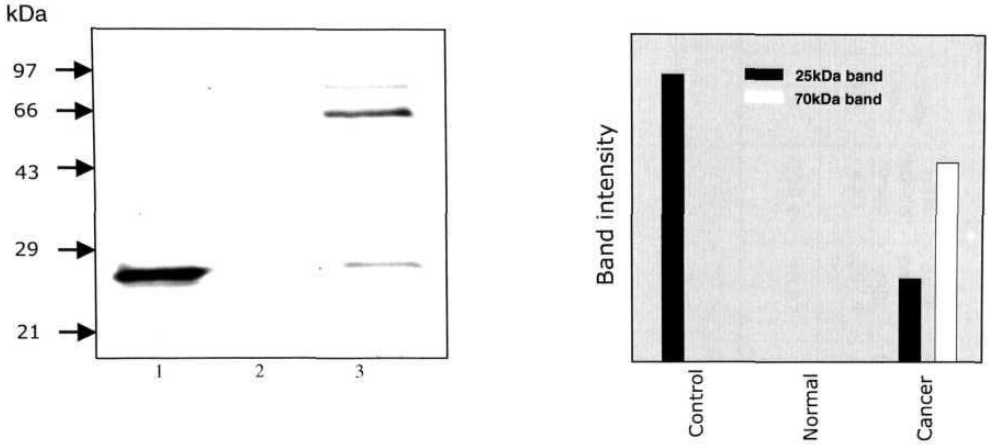
In all these blots with different class specific antibodies of GSTs, a band at the higher molecular weight of about 70kDa was detected. The appearance of a band at this higher molecular weight suggests a possible cross reactivity of some other protein which is co-eluted along with GSTs in affinity column chromatography or it could be a new form of GSTs which is seen specifically in cancerous breast tissue.

Fig. 3. 4. Western blot analysis of GSTs probed with GST mu class antibodies



Affinity purified GSTS were separated on 12% SDS-PAGE from human breast cancer and normal tissues, transferred on to nitrocellulose membrane and probed with mu class GST antibodies. Lane 1: human fetal liver GSTs, Lane 2: human breast normal tissue GSTs, Lane 3: human breast cancer tissue GSTs. The relative intensities of each band of 25kDa band are represented as solid bars and the 70kDa protein band as empty bars.

Fig. 3. 5. Western blot analysis of human breast cancer and normal tissue GSTs probed with GST pi class antibodies



Affinity purified GSTS were separated on 12% SDS-PAGE from human breast cancer and normal tissues, transferred on to nitrocellulose membrane and probed with mu class GST antibodies. Lane 1: human fetal liver GSTs, Lane 2: human breast normal tissue GSTs, Lane 3: human breast cancer tissue GSTs. The relative intensities of each band of 24kDa band are represented as solid bars and the 70kDa protein band as empty bars.

Table 3.1: Typical GSH affinity column purification profile of cytosolic GSTs from breast cancer tissues.

Source of protein	Protein (mg/mL)	Activity (U/mL)	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield %	Fold
1,05,000 X g supt.	3.071	4.3	430	307.1	1.4	100	1
GSH affinity purified	0.57	11.97	335.4	15.97	21	78	15

Table 3.2: Typical GSH affinity column purification profile of cytosolic GSTs from normal breast tissues.

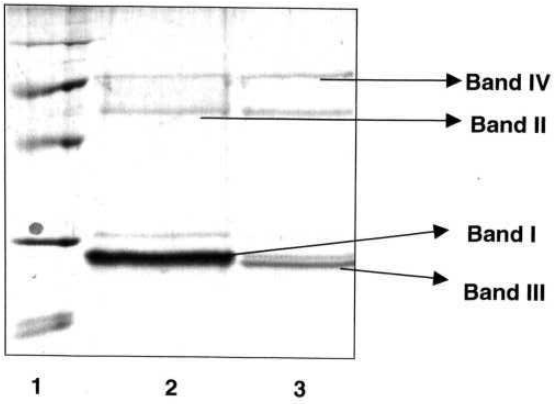
Source of protein	Protein (g/mL)	Activity (U/mL)	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield %	Fold
1,05,000 X g supt.	1.704	1.875	225	204.5	1.1	100	1
GSH affinity purified	.485	6.468	155.25	11.65	13.32	69	12

MALDI analysis:

In all the Western blots analyzed with GST antibodies, there was a signal at a molecular weight of approximately 70kDa along with the signal at 24-29kDa. The appearance of the signal at higher molecular weight (70kDa) was intriguing and prompted further analysis.

The purified GSTs were resolved on 12% SDS-PAGE and the coomassie stained protein bands were taken from the gel and analyzed on MALDI-TOF to identify the protein, based on peptide mass fingerprints (PMF). The protein bands used for analysis were represented in figure (Fig. 3.6).

Fig. 3.6. SDS-PAGE pattern showing the bands taken for MALDI analysis



Affinity purified GSTs from cancerous (lane 2 and 3) tissues of breast cancer patients were separated on 12% SDS-PAGE. The bands represented as I, II, III and IV were eluted from the gel and analyzed by MALDI-TOF for their identification through molecular weight and p*I* values. Lane 1: molecular weight marker. Lane 2 and 3: GSH affinity column purified GSTs from breast cancer tissues.

Four protein bands at a molecular weight range of 24-28kDa and 65-70kDa were analysed on MALDI-TOF. The band-I was identified as GSTM1 with an exact molecular weight of 25.7kDa and p/ of 6.2 (Fig. 3.7 and 3.8). Band-II was analysed as a hypothetical protein of yeast with a molecular weight of 44.1kDa and p/ 5.8 (Fig. 3.9 and 3.10). Band-III was analysed as GST protein with a molecular weight of 23.1kDa and p/ of 6.7 (Fig. 3.11). This protein band was also showing similarity with RAS-LIKE protein (Fig. 3.12).

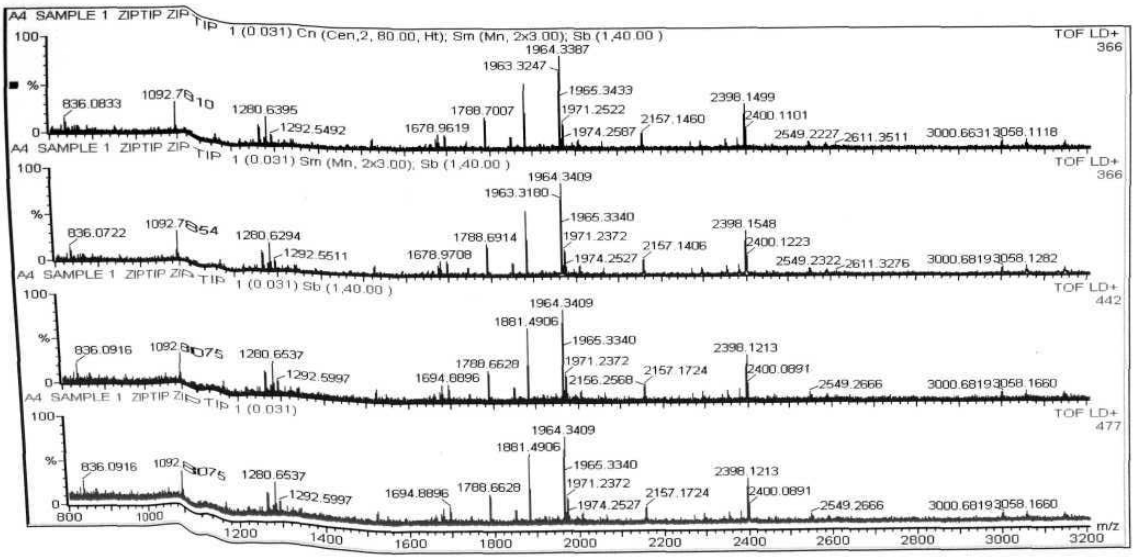
The protein band at a higher molecular weight of approximately 70kDa was also analyzed on MALDI. Interestingly, this protein band showed peptide fragments of GSTM1 (Fig. 3.13 and 3.14) along with another protein which was analyzed as synaptotagmin V (Syt V) (Fig. 3.15 and 3.16) with a molecular weight of 42.9kDa and p/ of 9.3 suggesting that this is an aggregation of GSTM1 with synaptotagmin V.

RT-PCR analysis:

The variation in the expression of GSTs in breast cancer tissues compared to the corresponding normal tissues was analyzed at mRNA level using reverse transcriptase polymerase chain reaction (RT-PCR) analysis. RNA was isolated from the tissue samples as mentioned in the methodology. Specific primers for different classes of GSTs (GSTM1, GSTT1 and GSTP1) along with glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal control were used in a multiplex RT-PCR to amplify the cDNA of the tissue samples.

The RT-PCR analysis of various individuals was done. Multipex RT-PCR

Fig. 3.7. Peptide mass fingerprints generated on MALDI analysis of Protein band I from Fig. 3.6



Peptide mass fingerprints (PMF) were obtained on MALDI analysis of protein band I (fig. 3.6) from human breast cancer tissue. The chromatogram shown is baseline corrected and normalized.

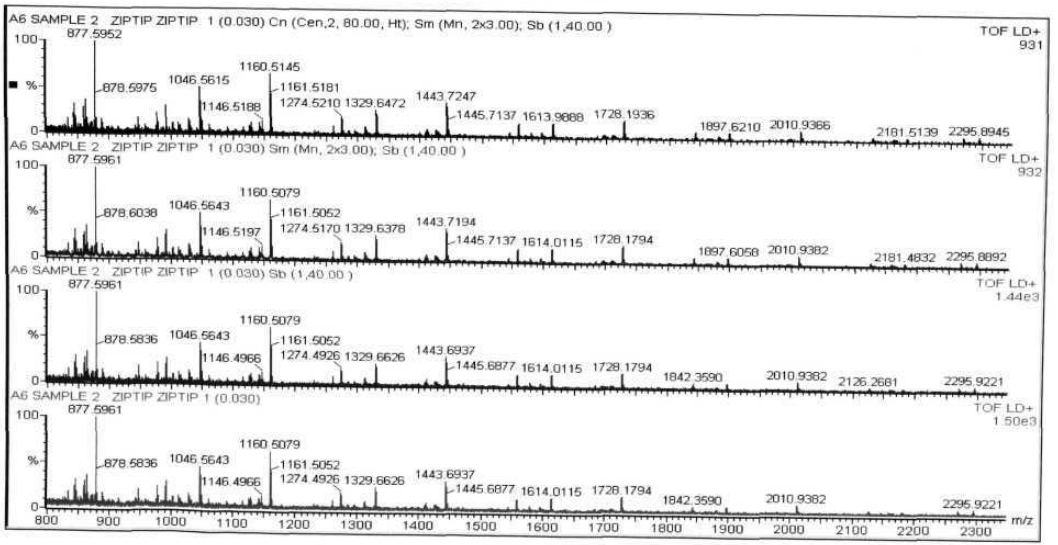
Fig. 3. 8. Summary data of GST protein band I as analyzed on MALDI (Fig. 3.7)

	MOWSE Score	#/45 (%) Masses Matched	% Cov	% TIC	Mean Err ppm	Data Tol ppm	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	1.311e+12	26 (57)	84.0	86.7	0.438	55.7	49328	25712/6.2	P09488	HUMAN	Glutathione S-transferase Mu 1 (GSTM1-1) (HB subunit 4) (GTH4) (GSTM1a-1a) (GSTM1b-1b) (GST class-mu 1)
2	4.119e+05	15 (33)	89.0	93.3	7.89	55.3	107521	25562/5.6	Q03013	HUMAN	Glutathione S-transferase Mu 4 (GSTM4-4) (GTS-Mu2) (GST class-mu 4)
3	3488	13 (28)	99.0	75.6	-1.33	49.0	3113	25693/6.9	P46439	HUMAN	Glutathione S-transferase Mu 5 (GSTM5-5) (GST class-Mu 5)
4	2099	16 (35)	80.0	77.8	7.59	51.9	3176	28181/5.8	P51580	HUMAN	Thiopurine S-methyltransferase (Thiopurine methyltransferase)
5	1019	15 (33)	55.0	95.6	1.50	63.5	121924	98602/8.7	Q61824	MOUSE	ADAM 12 precursor (A disintegrin and metalloproteinase domain 12) (Meltrin alpha)

49328	x x x x x x x x x x x x x x x x x x . x x x x x . x x x x x x x x x x . x x x x x . x . .
107521	x x x x x x x x x x x x x x x x x . x x x x x x x x x x x x x x x x x x x . x x x x .
3113	x x x x x x x x x x x x x x x x x . x x x x x . x . x x x x x . x x . x x . . x . . . .
3176	x x . x x x x x x x x x x x x x x . x x x x . x x x x x x x x x x . x x x . . . . . .
121924	x . x x x x x x x x x x x x x x x x x x x x x x x x x x x x x x x x x x x . x x x x x

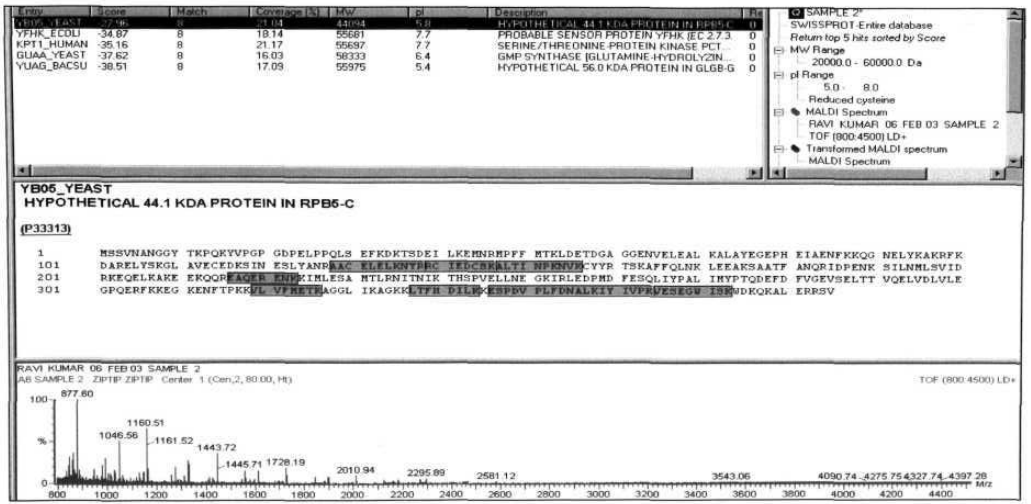
Based on PMF generated on MALDI-TOF, the band I (fig. 3.6) was identified as GSTM1 with a molecular weight of 23.1 and p*I* value of 6.2.

Fig. 3. 9. Peptide mass fingerprints generated on MALDI analysis of Protein band II from Fig. 3.6



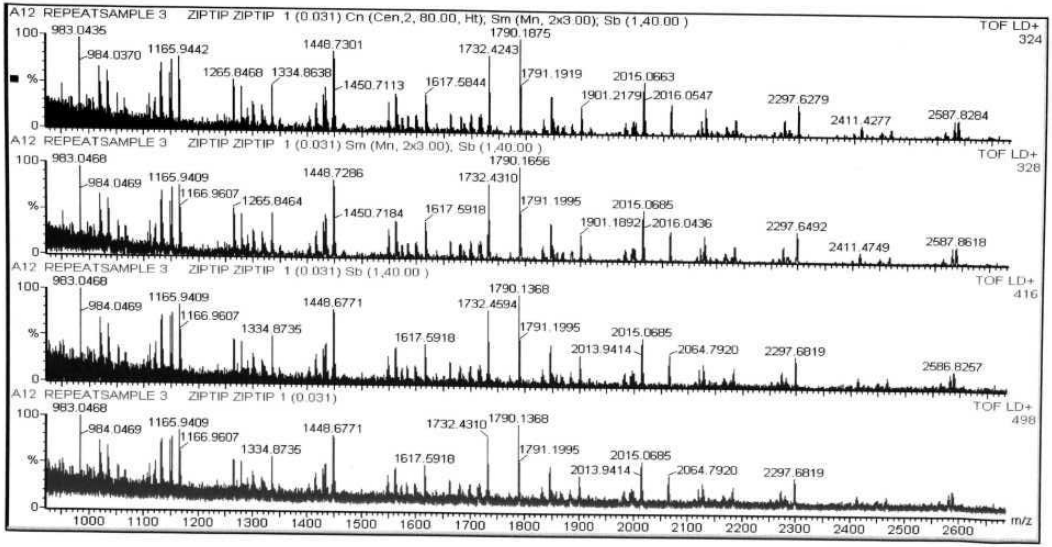
Peptide mass fingerprints were obtained on MALDI analysis of protein band II (fig. 3.6) from human breast cancer tissue. The chromatogram shown is baseline corrected and normalized.

Fig. 3. 10. Summary data of GST protein band II as analyzed on MALDI (Fig. 3.9)



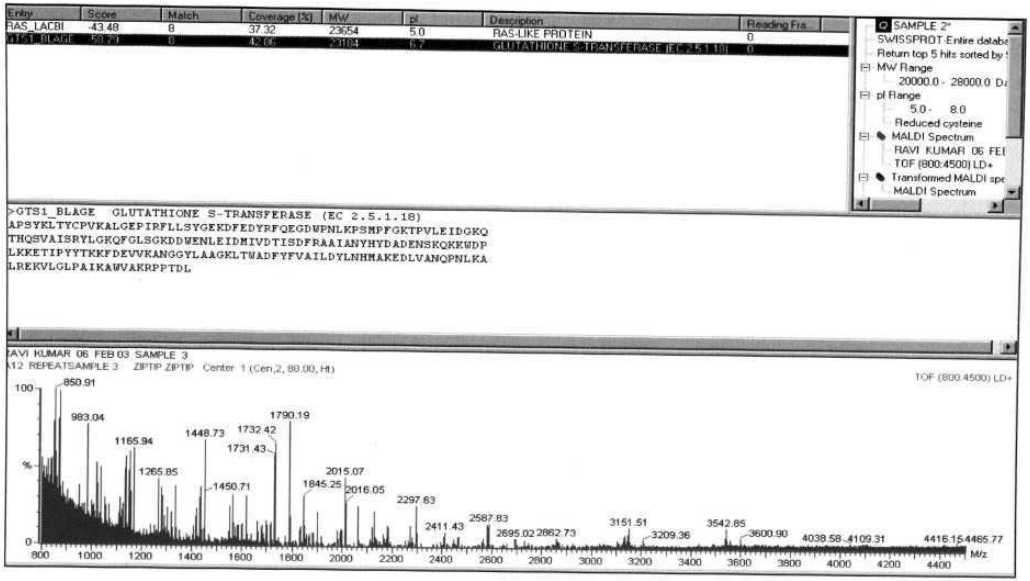
Based on PMF generated on MALDI-TOF, the band II (fig. 3.6) was identified as hypothetical protein with a molecular weight of 44.1 and p*I* value of 5.8.

Fig. 3. 11. Peptide mass fingerprints generated on MALDI analysis of Protein band III from Fig. 3.6



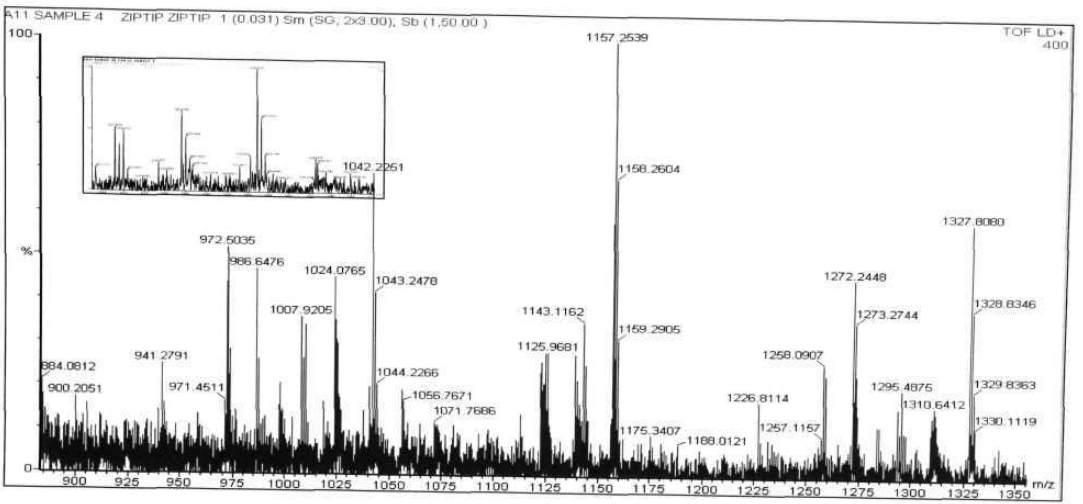
Peptide mass fingerprints were obtained on MALDI analysis of protein band III (fig. 3.6) from human breast cancer tissue. The chromatogram shown is baseline corrected and normalized.

Fig. 3. 12. Summary data of GST protein band IIIas analyzed on MALDI (Fig. 3.11)



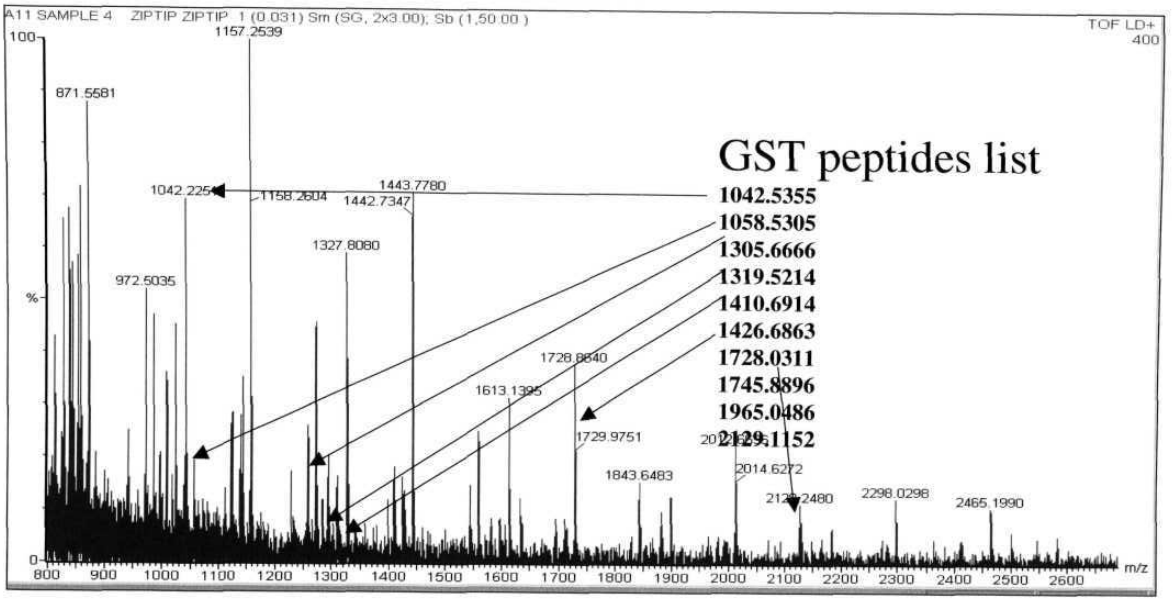
Based on PMF generated on MALDI-TOF, the band III (fig. 3.6) was identified as glutathione S-transferase protein with a molecular weight of 23.1 and p*I* value of 6.7.

Fig. 3. 13. Peptide mass fingerprints generated on MALDI analysis of Protein band IV from Fig. 3.6



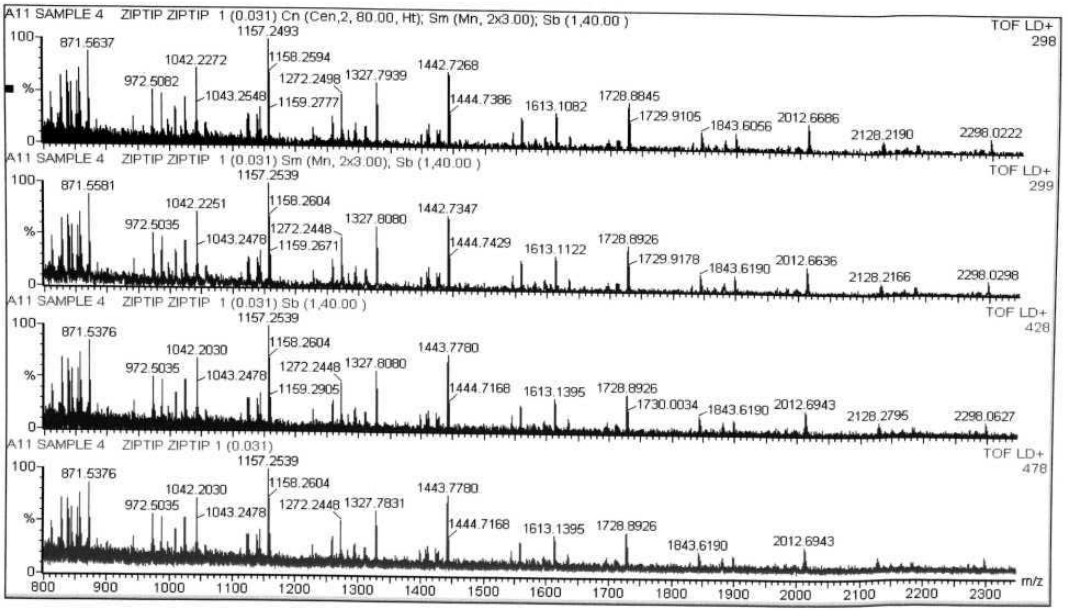
Peptide mass fingerprints were obtained on MALDI analysis of protein band IV (fig. 3.6) from human breast cancer tissue. The chromatogram shown as inset represents the PMF generated by GSTM1from band IV.

Fig. 3. 14. Peptide mass fingerprints generated on MALDI analysis of Protein band IV from Fig. 3.6



Peptide mass fingerprints were obtained on MALDI analysis of protein band IV (Fig. 3.6) from human breast cancer tissue. PMF generated by GSTM1from band IV are represented.

Fig. 3. 15. Peptide mass fingerprints generated on MALDI analysis of Protein band IV from Fig. 3.6



Peptide mass fingerprints were obtained on MALDI analysis of protein band IV (fig. 3.6) from human breast cancer tissue. The chromatogram shown is baseline corrected and normalized.

Fig. 3. 16. Summary data of GST protein band III as analyzed on MALDI (Fig. 3.13)

Data Set 1 Results

MS-Fit search selects **354** entries (results displayed for top **5** matches).

Results Summary

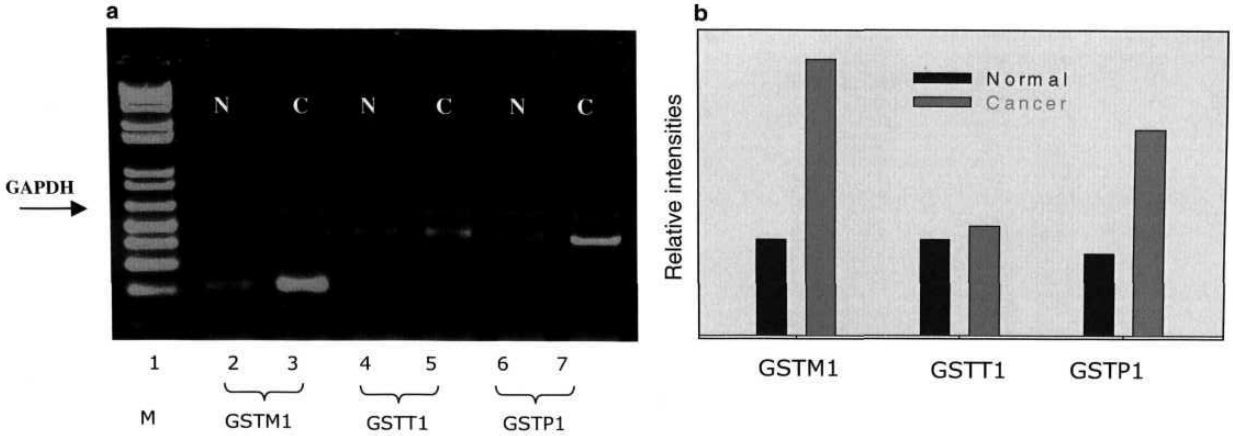
	MOWSE Score	#/10 (%) Masses Matched	% Cov	% TIC	Mean Err ppm	Data Tol ppm	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	75.3	4 (40)	47.0	60.0	-7.45	80.9	38547	42959/9.3	Q00445	HUMAN	Synaptotagmin V (SytV)
2	75.0	4 (40)	39.0	60.0	-5.26	84.7	35671	43127/9.4	P47861	RAT	Synaptotagmin V (SytV)
3	75.0	4 (40)	48.0	60.0	-11.2	73.9	96585	43130/9.6	Q9R0N5	MOUSE	Synaptotagmin V (SytV)
4	29.9	4 (40)	29.0	60.0	-11.3	114	21552	33638/6.7	Q9MZ03	RABIT	ADP-ribosyl cyclase (Cyclic ADP-ribose hydrolase) (cADPr hydrolase) (CD38 homolog)
5	16.5	3 (30)	40.0	50.0	-24.3	84.9	3568	27246/8.9	Q70351	RAT	3-hydroxyacyl-CoA dehydrogenase type II (Type II HADH) (Endoplasmic reticulum-associated amyloid beta-peptide binding protein)

Based on PMF generated on MALDI-TOF, the band III (fig. 3.6) was identified as synaptotagmin V protein with a molecular weight of 23.1 and p*I* value of 6.7.

was arranged as different sets i.e. GSTM1 + GAPDH, GSTT1 + GAPDH, and GSTP1 + GAPDH. These three sets of multiplex reactions were conducted in different patients. The expression study of six different samples (cancerous and normal) was analysed and their average showed an elevation in the expression of GSTM1 and GSTP1 (Fig. 3.17a). Lane 1 shows the marker (100bp) ladder, lane 2 shows the expression of GSTM1 in normal tissues and lane 3 shows the expression of GSTM1 in cancerous tissues. Lane 4 represents the expression of GSTT1 in normal tissues which is not different from that of cancer tissues represented in lane 5. The overexpression of GSTP1 in cancerous tissues is represented in lane 7 which is significantly higher compared to the corresponding normal tissue (lane 6). The relative intensities of these products in cancerous and normal tissues are represented in the form of bar diagrams (Fig. 3.17b).

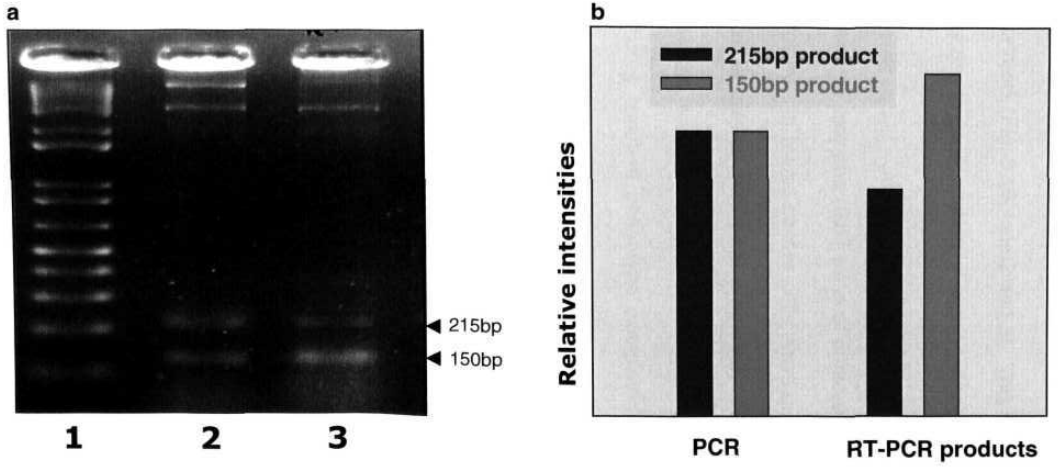
Among the various samples analyzed with cDNA, sample number 3 alone showed a double band with the GSTM1 primer (Fig. 3.18, Lane 3). The products were of molecular weight of 215bp which is of expected size and the other one is of 150bp. This 150bp fragment was thought to be a non-specific amplification product. To further confirm that if this 150bp product could be amplified with genomic DNA as template, direct PCR was conducted with genomic DNA (Fig. 3.18, lane 2). As shown in the figure two bands of 215bp and 150bp amplified from the genomic DNA also. This suggests that this could be the amplification of some non-specific gene or the truncated product of GSTM1 gene. However, among all the tissue samples analyzed for the expression of GSTM1 at mRNA

Fig. 3. 17. RT-PCR analysis of GST mu, theta and pi classes in cancerous and normal tissues



(a) RT-PCR analysis of GSTM1, GSTT1 and GSTP1 genes of human breast cancer and normal tissues. The RT-PCR products were separated on 1% agarose gel electrophoresis showing the over expression of GSTM1 and GSTP1 in cancerous tissues compared to the corresponding normal tissues. Lane 2 and 3 are the RT-PCR products of GSTM1 (215bp), from normal and cancer tissues respectively. Lane 4 and 5 are the RT-PCR products of GSTT1 (480bp) from normal and cancer tissues respectively. Lane 6 and 7 are the RT-PCR products of GSTP1 (415bp) from normal and cancer tissues respectively. Glyceraldehyde phosphate dehydrogenase gene primers were used as internal control (550bp). (b) The relative intensities of each band is represented as solid bars

Fig. 3. 18. PCR and RT-PCR analysis of GSTM1 gene in patient # 3



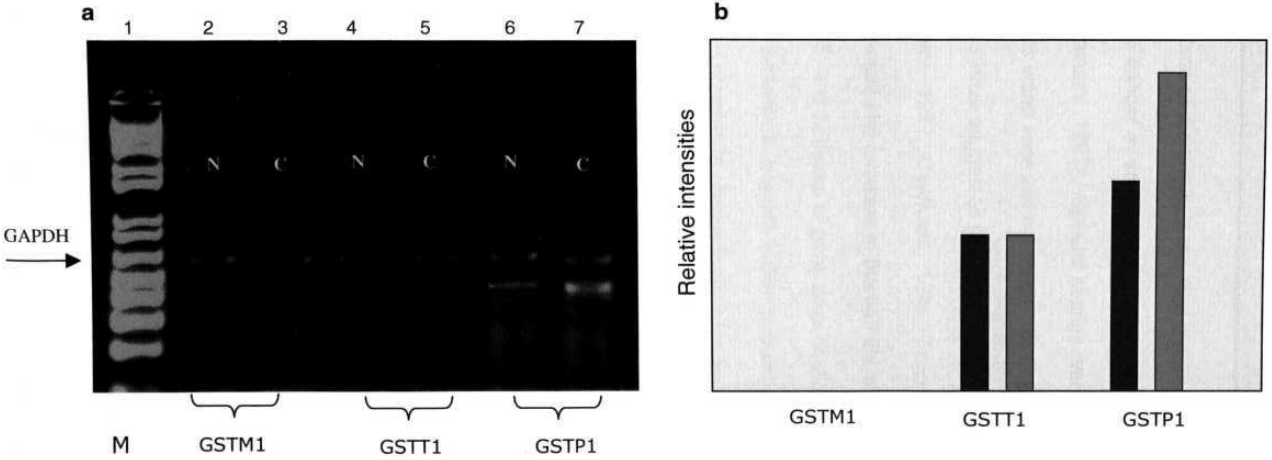
(a) Agarose gel electrophoresis of PCR and RT-PCR product of GSTM1 from breast cancer tissue of patient number 3. The products were of sizes corresponding to 150bp and 215bp. Lane 1: Mol. Wt. Marker, Lane 2: Genomic DNA PCR product of GST M1, Lane 3: RT-PCR product of GST M1. (b) The relative intensities of each band is represented as solid bars.

level, a certain number (25%) of samples showed no product amplification with GSTM1 specific primers (Fig. 3.19, lane 2 and 3). This evoked interest in finding out whether this is due to gene deletion or non-expression.

Discussion

The pi class GST isozyme (GST pi) is of particular interest in the study of breast cancer etiology. In breast cancer cells, increased GSTP1 expression is associated with other altered biological properties. In some cultured breast cancer cells, repeated exposure of these cells to certain anticancer agents results in a multidrug resistant phenotype associated with increased GSTP1 expression and other altered biochemical properties, including loss of estrogen receptors (ER) expression (Baptist, 1986). The determination of GST levels in blood has been proposed to be a marker of tumor burden in general, where as level of the pi isozymes has been identified as a prognostic factor for breast cancer patients receiving neo-adjuvant therapy (Kelley, 1994). The role of GST pi in cancer tissues is generally implicated in multidrug resistance. It was suggested that it is not the overexpression of GST per se but the interplay between GSH/GST and glutathione conjugate efflux pumps that result in increased resistance to alkylating anticancer drugs such as thiotepa. The coordinated expression of MDR and GST pi genes in breast cancers suggest that they may share common regulatory elements for their expression (Morrow, 1998) Consequently, the variation in the expression of various classes of GST with a special emphasis on GST pi is of considerable importance in breast cancers.

Fig. 3. 19. RT-PCR analysis of GST mu, theta and pi classes in cancerous and normal tissues



a) RT-PCR analysis of GSTM1, GSTT1 and GSTP1 genes of human breast normal and cancer tissues. The RT-PCR products were separated on 1% agarose gel electrophoresis showing no product of GSTM1 in both normal and cancer tissue and over expression of GSTP1 in cancerous tissues compared to the corresponding normal tissue. Lane 2 and 3 are the RT-PCR products of GSTM1 (no product seen) from normal and cancer tissues respectively. Lane 4 and 5 are the RT-PCR products of GSTT1 (480bp) from normal and cancer tissues respectively. Lane 6 and 7 are the RT-PCR products of GSTP1 (415bp) from normal and cancer tissues respectively. Glyceraldehyde phosphate dehydrogenase gene primers were used as internal control. b) The relative intensities of each band is represented as solid bars inset.

Carcinoma of the breast is the third most common in the world and second most common cancer in south India and is the cause for highest morbidiy and mortality (Gajalaxmi, 1997). Several studies were conducted on GSTs in breast cancer patients world wide but not much has been studied in Indian populations. Several reports have suggested the elevated expression of GSTs in spontaneous cancers (Mitaka, 1987, Oyamado, 1998, Tsuchida, 1989). The induction is attributed to factors like increase in the half life of the protein and mRNA stability (Hongxie, 1995) and because of gene amplification (Chao, 1987). Our results on breast cancer patients in a south Indian population showed specific induction of GST pi and the proteins in cancer tissues (as revealed by Western blot analysis) compared to the corresponding normal tissues. There are several studies which suggested the induction of GST pi isoform in a variety of malignancies including carcinoma of the colon (Ramakrishna, 1998), lung, kidney, ovaries, pancreas, oesophagus and stomach (Harrison, 1989; Shiratori, 1987; Dillio, 1987; Di Ilio 1988; Eimoto, 1988; Shea, 1988; Moscow, 1989; Peters, 1989)

Recent report suggests that GSTs may play a role in the regulation of both cellular proliferation and apoptosis (Bernardini, 2000). It was also shown that GST P1-1 is the most widely distributed extra hepatic isozymes (Chang 2001). The present study reports the overexpression of GST pi and mu at the level of mRNA in breast cancer tissues as compared to the corresponding normal tissues. Similarly induction is reported in GST pi at mRNA level in renal cell carcinoma of rats (Tanaka, 1988), and humans (Di Ilio, 1990) and squamous cell

carcinoma of head and neck (Moscow, 1989). Similar expression of all three classes of GSTs was reported in breast tissue with GSTpi and mu class enzymes preponderated (Forrester, 1990).

From the results obtained in the present study, overexpression of mu and pi classes GSTs was also observed by RT-PCR analysis. Another important finding of the present study is the association of GSTM1 with synaptotagmin V (Syt V) protein of molecular weight of 42.9kDa. Synaptotagmin now has an established post docking role in secretion and also appears to be a key component with the idea that synaptotagmin is a major Ca2++ sensor that triggers release. This family may actually consist of 19 isoforms, some of which exhibit alternative splicing (Craxton, 2001). Interestingly, synaptotagmin proteins have been detected in tissues outside the nervous system. For example, synaptotagmin XIII has been found in sperm heads (localized to acrosomes) (Michaut, 2001; Hutt, 2002) and in the kidney (Kishore, 1998). Similarly synaptotagmin VII has been detected not only in brain but also in heart, lung and spleen (Li, 1995; Sugita, 2001).

Its presence in breast tissue especially cancerous tissues of breast has not been reported so far. Its role in nervous system has been well demonstrated but not much is known about its role in other tissues. It would be interesting to study indepth about its role in breast cancers.

It is well known that GSTs conjugate with specific proteins and modulate their function. GST pi is known to play a major role in regulating N-terminal jun

kinase (JNK) activity in normal cells by forming a complex of GST-JNK (Victor, 1999). This GST-JNK complex is broken down upon UV irradiation or H_2O_2 treatment. This finding ascertained a novel function to GSTs apart from its regular detoxification and ligand binding activities. This forms the first report on the GST mu complexing with synaptotagmin V like protein in breast cancers. It will be interesting to probe further on the identification of this protein and its function upon complexing with GST mu in breast cancers.

It was found in few samples that the gene product of GSTM1 was not amplified in RT-PCR analysis, suggesting the possibility of gene deletion in these patients. In order to test this, further studies were taken up on GST gene deletions and the data presented in the next chapter.

Thus the present study reveals the induction of GST pi and GST mu in breast cancer tissues when compared to their corresponding normal tissues. Also the studies show the conjugation of GSTM1 with synaptotagmin V like protein of molecular weight of 42.9kDa and p/ of 9.3. The precise role of this protein in cancers would be of diagnostic value. Further detailed studies on the identification of this protein and understanding its role in complexing with GSTM1 and its overexpression only in the breast cancer tissues would throw light in the understanding of the etiology of breast cancers. The present study also shows lack of expression of GSTM1 in certain patients, indicating the possible gene deletion. Further studies on genomic DNA, however, are required to analyze the frequency of GST gene deletions in breast cancerous and normal healthy

subjects, to understand the role of GSTs in the etiology of breast cancers.

CHAPTER IV

Title: Genetic polymorphism of GSTs in south Indian population and breast cancer susceptibility.

Introduction:

Many compounds in their natural or metabolized forms are capable of interacting with DNA and impair their integrity and ultimately leading to DNA damage. Accumulation of these DNA damages added to spontaneous DNA replication errors that are not corrected by DNA repair system, result in irreversible mutations. Accumulation of these genetic alterations would lead to neoplastic transformation and/or progression to cancers. It has been reported that about 80% of human cancers arise as a consequence of environmental exposure (Doll et al., 1981). Though lung and liver are the primary targets of environmental contaminants, there are increasing evidences for the susceptibility of other tissues, including breast tissue.

Glutathione S-transferases (GSTs) are a group of multigene and multifunctional proteins involved in detoxifying xenobiotic electrophilic compounds by catalyzing their conjugation to glutathione. The GSTs in humans are primarily grouped under three main classes – GST Mu, GST Theta and GST Pi. Among these gene polymorphism is usually associated with GSTM1 and GSTT1 in humans. In central Europe GSTM1 is homozygously deleted in about 50% and GSTT1 in 10% of caucasian individuals (Hayes et al, 1995). .

Individuals with GSTM1 deficiency show a greater level of DNA-damage following carcinogen exposure, as determined by sister chromatid exchange

(SCE) and formation of DNA-adducts (Kato et al., 1995). Several studies have implicated GSTM1 gene deficiency to susceptibility of individuals to a range of cancers (Hayes and Pulford, 1995). Smoking and GSTM1 null genotype were found to be risk factors for squamous cell carcinoma (Bernadette Sehoket, 1998). The GSTM1 homozygous null genotype was associated with an increased risk of developing breast cancer, principally in association with postmenopausal breast cancer (Helzlsouer, 1998). The risk of breast cancer increased as number of putative high-risk genotypes increased for a combined genotype GSTM1 null and GSTP1 valine heterozygosity (Watson, 1998). The present study on breast cancer patients has shown higher activity levels of GSTs, specifically GST pi and GST mu, in most of the cancer tissues compared to the adjacent normal tissues. However, in some patients no signals of GSTM1 were observed, as indicated through RT-PCR analysis, indicating the probable gene deletions. In order to find any possible correlation between GSTs gene deletion and the incidence of breast cancers, further studies were undertaken to analyze the frequency of GST gene deletions in breast cancer patients and age and sex matched healthy volunteers

Results

GST gene polymorphisms play a major role in enhanced susceptibility to various cancers, specifically of lung and liver. In the present study, GST gene polymorphisms, specifically GSTM1 gene was undertaken in the breast cancer patients coming to local hospitals in Hyderabad, South India. Normal healthy

volunteers in Hyderabad served as controls for the study. About sixty breast cancer patients and fifty eight age and sex matched normal individuals formed the study group in the present study.

Genomic DNA isolation:

The genomic DNA from the blood samples of the breast cancer patients and healthy volunteers was isolated for the analysis. The molecular weight of the genomic DNA isolated from different individuals was approximately 10kb in size. DNA was quantified on spectrophotometer by monitoring the UV absorbance at 260nm. The samples having the absorbance ratio (260/280nm) greater than 1.7 were used in further experiments. The samples showing the absorbance ratio less than 1.7 were used to re-extract the DNA using phenol: chloroform.

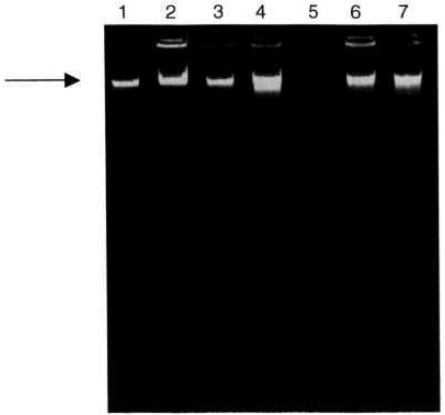
Agarose gel electrophoresis:

The DNA samples from breast cancer patients and healthy volunteers were subjected to agarose gel electrophoresis to check the purity and quantify the samples (Fig. 4.1). The isolated genomic DNA (50ng) from each sample was used for PCR analysis. The PCR primers of GSTM1, GSTT1 and GSTP1 as mentioned in the methodology were used for the amplification of respective gene fragments.

PCR analysis:

GSTM1 and GSTT1 were amplified in a multiplex PCR with the respective primers together in a single tube. The products were analyzed on agarose gel electrophoresis. The product of GSTM1 was of 215bp and that of GSTT1 was

Fig. 4. 1. Agarose gel electrophoretic pattern of genomic DNA



Genomic DNA isolated from blood samples of breast cancer patients and normal individuals checked on 1% agarose gel electrophoresis. Lane 1: normal individual's DNA 500ng. Lane 2 to 7 Genomic DNA samples isolated from breast cancer patients blood samples.

480bp (Fig. 4.2 and 4.3). The frequency of GSTT1 gene deletions in cancer patients was relatively less compared to those of the GSTM1 gene deletions. The samples which showed no product on multiplex PCR were checked again by repeating individually with the primer set of only one gene. The PCR analysis of GSTM1 gene revealed higher frequency of gene deletions (36%) in breast cancer patients (n=60) (Fig. 4.2) compared to 18% deletions in age and sex matched healthy volunteers n=58 (Fig. 4.3). The values are highly significant at $P<0.05$ level. The PCR analysis of GSTT1 gene also revealed higher frequency of gene deletions in breast cancer patients i.e. 7%, n=60 compared to 3% deletions in age and sex matched healthy volunteers (n=58). The PCR analysis of GSTP1 gene was also performed in all these samples but no case of GSTP1 gene deletion was observed. In all the samples the GSTP1 gene was present i.e. in patients as well as healthy volunteers. GSTs enzymatic activity in GSTM1 deleted patients was calculated and data represented in Figure 4.4. As shown in the figure total GST activity in cancer tissues of GSTM1 gene deleted breast cancer patients was significantly lower compared to the corresponding adjacent normal breast tissue. This is quite different from that in GSTM1 non-deleted patients, where in total GST activity in cancer tissues was significantly higher compared to that in normal tissue.

Apoptotic DNA ladder formation:

In some of the breast cancer DNA samples, typical apoptotic DNA laddering pattern was seen on agarose gel electrophoresis (Fig. 4.5).

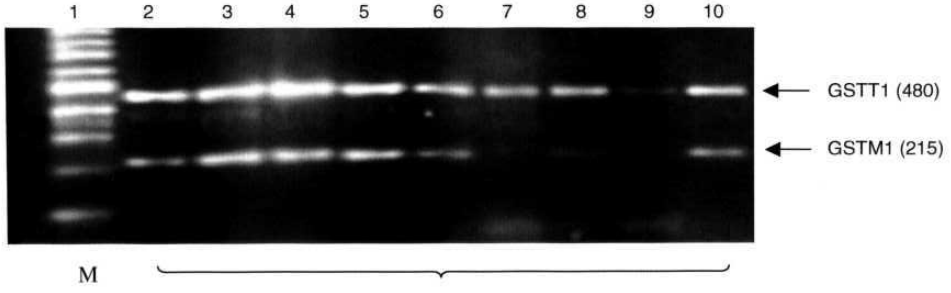
SNPs in GST genes of breast cancer tissue

In view of the high frequency of SNPs reported in GSTP1 gene, further studies were undertaken to identify single nucleotide polymorphisms in this gene. The genomic DNA of breast cancer tissue samples was isolated and separated on agarose gel electrophoresis. These samples on agarose gel showed DNA at a molecular weight of approximately 10kb (Fig. 4.6). From these samples 50ng of genomic DNA was used for PCR analysis using GSTP1 primers.

The PCR product of GSTP1 (size 432bp) was subjected to single stranded conformational polymorphism (SSCP) gel, as mentioned in the methodology, to analyze point mutations if any. The products were resolved at a molecular weight higher (700bp to 1000bp) than that of the double stranded products (432bp). Also on 7% acrylamide gel electrophoresis the PCR products resolved into three bands in normal tissue sample (lane 4) and into four bands in cancerous tissue sample (lane 5). The resolution of the fourth band in the cancerous sample indicates the mutation in that sample (Fig. 4.7).

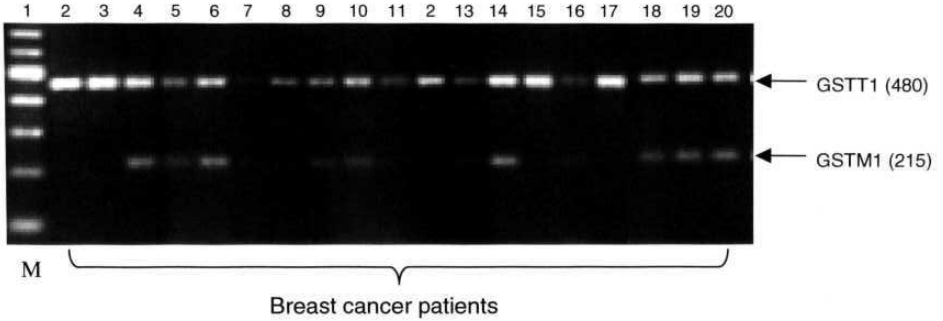
Further to confirm the point mutations, the sample which showed extra band on SSCP gel was subjected to sequencing analysis. The gene sequencing of these samples has shown point mutations at five positions (Fig. 4.8). The specific mutations are addition of Thymine, deletion of guanine and substitution of Thymine for purines (T → A/G)

Fig. 4. 2. PCR analysis of GST genes in normal individuals



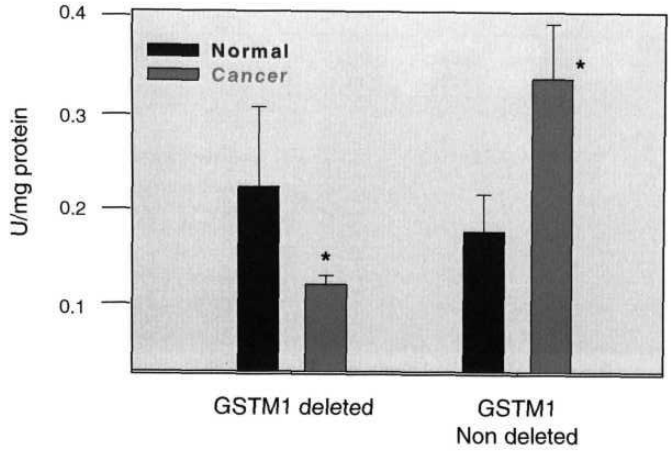
Multiplex PCR analysis of GSTM1 and GSTT1 genes from the blood samples of normal healthy volunteers showing gene deletions of GSTM1 gene (lanes 7 and 9). The molecular sizes of GSTM1 gene product is 215bp and that of GSTT1 is 480bp. Frequency of gene deletions of GSTM1 were 18% (n=58).

Fig. 4. 3. PCR analysis of GST genes in breast cancer patients



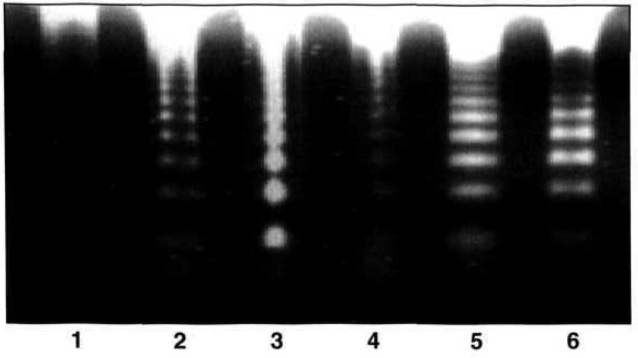
Multiplex PCR analysis of GSTM1 and GSTT1 genes from the blood samples of breast cancer patients showing gene deletions of GSTM1 gene (lanes 2, 3, 8 13, 16 and 18). The molecular size of GSTM1 gene product is 215bp and that of GSTT1 is 480bp. Frequency of gene deletions of GSTM1 were 36% (n=60).

Fig. 4. 4. Total GST activity in normal and cancerous breast tissues of GSTM1 gene deleted and non-deleted patients



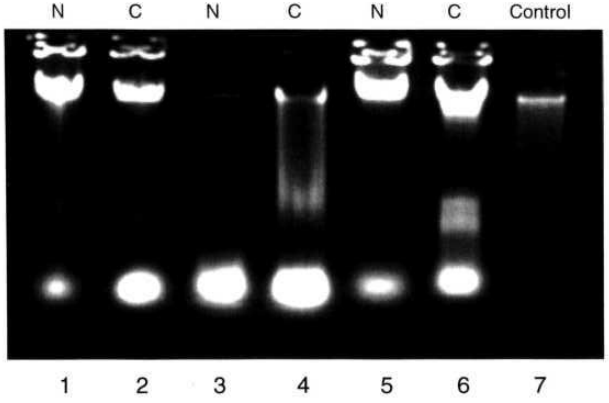
The GST enzyme activity measured in human breast cancer and normal tissues of patients with and without GSTM1 gene deletions. The activity was significantly higher in cancer tissues of patients having no gene deletion compared to those having GSTM1 gene deletion. The values are significant at $p<0.01$ (student's t-test).

Fig. 4. 5. Agarose gel electrophoresis of human genomic DNA isolated from blood samples of breast cancer patients



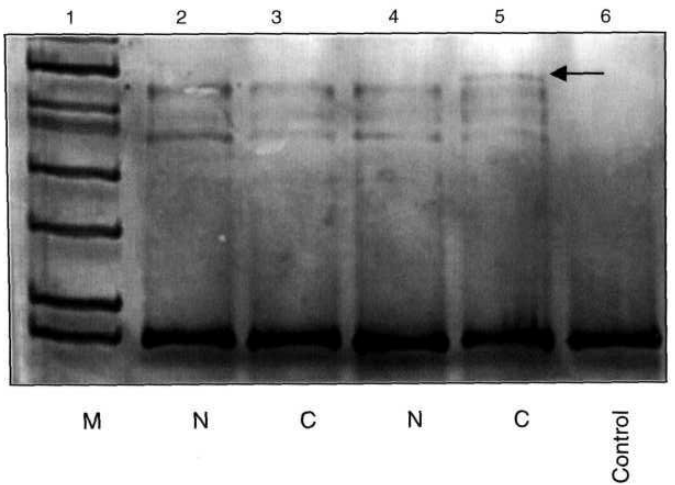
Agarose gel electrophoresis of human genomic DNA isolated from blood samples of breast cancer patients. Lane 1: Genomic DNA isolated from normal individual Lane 2 to 6: Genomic DNA isolated from blood of breast cancer patients showing apoptotic DNA fragmentation (ladder pattern).

Fig. 4. 6. Genomic DNA of breast cancer tissues



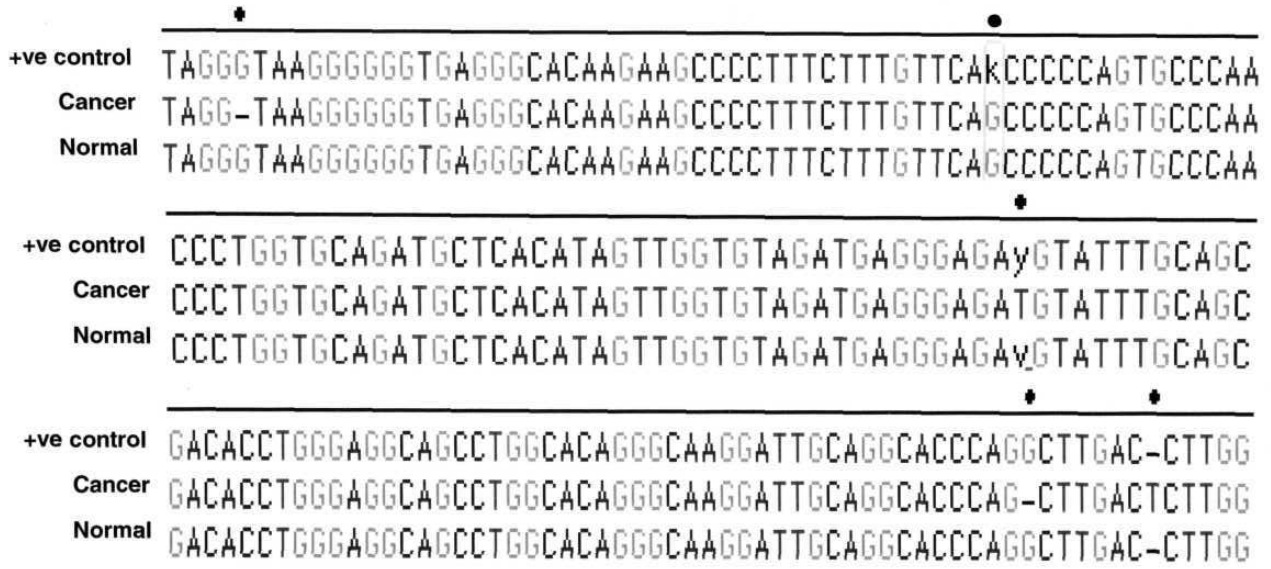
Genomic DNA, isolated from breast cancerous and corresponding normal tissues of patients, separated on 1% agarose gel electrophoresis. Lane 1,3 and 5 correspond to the normal tissue DNA. Where as lane 2,4, and 6 correspond to the cancer tissue DNA. Lane no. 7 represents the DNA isolated from blood sample of normal individual (50ng).

Fig. 4. 7. SSCP analysis



SSCP analysis of GSTP1 gene product (415bp) amplified through PCR. The PCR products of GSTP1 were denatured and separated on 7.5% acrylamide gel. Lane 1: Molecular weight marker. Lane 2 and 3: normal and cancer tissue samples of patient number 22. Lane 4 and 5: normal and cancer tissue samples of patient number 23. Lane 6: negative control.

Fig. 4. 8. DNA sequencing



DNA sequencing of GSTP1 gene from cancer tissue sample of patient number 23 showing point mutations. Mutations of Cancer tissue DNA is compared with normal tissue sequence and also the *consensus sequence* of DNA sample from Normal healthy individual's blood.

>PNT (DNA sequence of PCR product of GSTP1 gene from normal tissue)

5' GTA GTT TGC CCA AGG TCA AG

TAGGGTAAGGGGGGTGAGGGCACAAGAAGCCCCTTTCTTTGTTCAGCCCC

CAGTGCCCAACCCTGGTGCAGATGCTCACATAGTTGGTGTAGATGAGGGAG

AyGTATTTGCAGCGGAGGTCCTCCACGCCGTCATTCACCATGTCCACCAGG

GCTGCCTCCTGCTGGTCCTTCCCATAGAGCCCTGGGGTTGGGTGGAGGGG

AGCAGGCCGCGTGACTGGGCGCCTGATCAACACACAGTCACTGGGGATGG

GCCAACCAGGGGGCTGTCTGTGAGCCCCAGCCTGGGAGGAGGGGAAGAG

GATGTGCGTGGAAGGATGAGAGACTGCCACACAGCAGGTGCTCAGAGCTC

ACCTGACACCTGGGAGGCAGCCTGGCACAGGGCAAGGATTGCAGGCACCC

AGGCTTGAC_CTTGGAGCCACCTGAGGGGTAAG 3'

>PCT (DNA sequence of PCR product of GSTP1 gene from cancer tissue)

5' GTA GTT TGC CCA AGG TCA AG

TAGG_TAAGGGGGGTGAGGGCACAAGAAGCCCCTTTCTTTGTTCAGCCCCC

AGTGCCCAACCCTGGTGCAGATGCTCACATAGTTGGTGTAGATGAGGGAGA

TGTATTTGCAGCGGAGGTCCTCCACGCCGTCATTCACCATGTCCACCAGGG

CTGCCTCCTGCTGGTCCTTCCCATAGAGCCCTGGGGTTGGGTGGAGGGGA

GCAGGCCGCGTGACTGGGCGCCTGATCAACACACAGTCACTGGGGATGGG

CCAACCAGGGGGCTGTCTGTGAGCCCCAGCCTGGGAGGAGGGGAAGAGG

ATGTGCGTGGAAGGATGAGAGACTGCCACACAGCAGGTGCTCAGAGCTCA

CCTGACACCTGGGAGGCAGCCTGGCACAGGGCAAGGATTGCAGGCACCCA

GCTTGACTCTTGG AGCCACCTGAGGGGTAAG 3'

>PPT (GSTP1 DNA sequence of normal volunteer's blood)

5' GTA GTT TGC CCA AGG TCA AG

TAGGGTAAGGGGGGTGAGGGCACAAGAAGCCCCTTTCTTTGTTCAkCCCCC

AGTGCCCAACCCTGGTGCAGATGCTCACATAGTTGGTGTAGATGAGGGAGA

yGTATTTGCAGCGGAGGTCCTCCACGCCGTCATTCACCATGTCCACCAGGG

CTGCCTCCTGCTGGTCCTTCCCATAGAGCCCT*GGGGTTGGGTGGAGGGGA*

GCAGGCCGCGTGACTGGGCGCCTGATCAACACACAGTCACTGGGGATGGG

CCAACCAGGGGGCTGTCTGTGAGCCCCAGCCTGGGAGGAGGGGAAGAGG

ATGTGCGTGGAAGGATGAGAGACTGCCACACAGCAGGTGCTCAGAGCTCA

CCTGACACCTGGGAGGCAGCCTGGCACAGGGCAAGGATTGCAGGCACCCA

GGCTTGAC_CTTGG AGCCACCTGAGGGGTAAG 3'

Discussion

Molecular epidemiological studies of individuals with GSTM1 deficiency revealed a moderately increased risk for carcinomas of the lung, bladder and colon (Seidgard et al., 1990). In a population study in Italy a strong association between GSTM1 gene deletion and colon/breast cancers was reported (Sgambato et al., 2002). In the present study, a significantly higher frequency in the GSTM1 gene deletions was observed in breast cancer patients as compared to the age and sex matched normal individuals.

Mounting epidemiological evidence suggests that smoking may play a role in the etiology of breast cancer. In a case-control study, it was found that, individuals lacking GSTT1 gene were sensitive to benzo(a)pyrene diol epoxide

induced chromosomal aberrations. This may contribute to the risk of developing breast cancer, and such sensitivity may be modulated by both genetic and environmental factors (Xiong et al., 2001)

Apart from smoking related risk of breast cancers, other risk factors include alcohol-consumption. Higher incidence of breast cancers was observed in alcohol consuming premenopausal women lacking both GSTM1 and GSTT1 genes, when compared to those with both the genes (Park et al., 2000). These studies suggest the protective role played by GSTM1 and GSTT1 genes against benzo(a)pyrene/aldehyde radical mediated DNA damage.

In addition to xenobiotics, reactive oxygen species, specifically superoxide anion and hydroxyl radicals produced by the endogenous metabolism also play an important role in causing DNA damage. In breast carcinoma, many of these DNA lesions may be derived from hydroxy radicals and other ensuing lipid peroxides. The intrinsic peroxidase activity of GSTM1 (Hong, 1989) could play an important role in offering the protection from free radical and lipid peroxide-induced DNA damage.

Recent studies suggested that polymorphisms of CYP1A1, GSTM1 and NAT2 genes significantly enhance the frequency or the level of DNA adducts in the breast tissues of women having breast cancers, especially in smokers (Firozi et al., 2002). A significant increase in the risk of breast cancer in women was reported in the population with the combination of GSTM1 null, GSTP1 Ile/Ile, and GSTT1 null genotypes (Mitrunen et al., 2001). These studies suggest that

allelopolymorphism of GSTs is a significant modifier of breast cancer risk, in addition to other well known risk factors.

Apart from gene deletions there are single nucleotide polymorphisms (SNPs) in GST genes which might impair their function and eventually leading to onset of cancers due to lack of protection from radical mediated damages. SNPs were found in many important genes, as evidenced from the data mining of publicly available DNA sequences. SNPs in GSTP1, resulting in amino acid substitutions at codon 104 (Ile-Val) and 113 (Ala-Val) are also relatively frequent in the Caucasian population (Yang, 2002). In the present study on South Indian population also SNPs in GSTP1 gene were observed in human breast cancer patients. The results from SSCP analysis and DNA sequencing analysis have shown that GSTP1 is prone to gene mutations. The prominent among these mutations observed in the present study include single nucleotide additions, single nucleotide deletions and single nucleotide substitutions. They are addition of Thymine and deletion of Guanine and substitution of Thymine to Adenine (T→ A) or Guanine (T→ G). This increased frequency of SNPs in GSTP1 gene could make a product with altered enzymatic activity and thus making an individual sensitive to xenobiotic insults.

The above studies on GST gene polymorphisms, thus have revealed a significant correlation between the frequency of GSTM1 gene deletion and on set of breast cancers in South Indian population. The higher level of SNPs in GSTP1 was also observed in breast cancer patients, which could also be a

contributing factor for the early onset of breast cancers.

Since the patients coming to the hospitals in Hyderabad are drawn from places in and around Hyderabad, there is scope for these populations getting exposed to industrial pollutants, automobile exhausts and urban stress. In addition, the patients may be from the nearby tobacco belt (Vijayawada and Guntur which are approximately 200 km from Hyderabad), suggesting the possible impact of tobacco smoking on the incidence of breast cancers. Thus the exposure of the individuals to the industrial/urban pollutants, tobacco smoke and other stress factors, could be the contributing factors for the increasing incidence of breast cancers in the populations with GSTM1 gene deletions or SNPs in GSTP1 gene, where antioxidant/xenobiotic defences have been compromised. Further in depth field studies on the exposure of the population to environmental pollutants/industrial toxicants and urban stress factors would throw light on the increasing incidence of breast cancers in this particular part of South India.

Summary

Summary

Breast Cancer is one of the major cancers in women all over the world. It ranks the number one of the cancers in United States, with roughly 300,000 people affected per year (Jemal et al; 2002). Breast cancer occurrence is on raise and is the second most common cancer among women in India. There is higher incidence of breast cancers in urban centres, suggesting the possible impact of environmental pollutants and industrial toxicants on the incidence of breast cancers. The impact of the above factors will be much more in individuals with impaired detoxification systems and antioxidant defenses. The present study is, therefore, undertaken to analyze the cellular detoxification systems and antioxidant defenses in breast cancer patients attending to local hospitals in Hyderabad. The study is undertaken on 118 human subject, 60 breast cancer patients and in 58 age/sex matched healthy volunteers.

Among the antioxidant defense systems, glutathione (oxidized and reduced), glutathione reductase, glutathione peroxidase (Se-GPx and Non-Se-GPx) and glutathione S-transferases are predominant. These studies revealed higher levels of glutathione, glutathione reductases and GSTs with no significant changes in glutathione peroxidase activities in cancerous tissues compared to the corresponding adjacent normal tissues. Studies on other selected markers of cancers revealed increased expression of COX-2 and p53 with no changes in PPAR-γ and APAF-1 in cancer tissues compared to the corresponding normal tissues. Thus among the molecular markers studied, GSTs, COX-2 and p53 showed significant variation in the expression in cancer tissues compared to the

normal tissues. Of these, COX-2 is known to be involved in the development of multidrug resistance in individuals undergoing chemotherapy. GSTs, on the other hand, play an important role in the detoxification of xenobiotics as well as in the cellular antioxidant defenses. Hence further studies were undertaken on their purification and characteriztation from breast cancer and the adjoining normal tissues.

Purification of GSTs from normal and cancer tissues by GSH affinity column chromatography was done and separated on SDS-PAGE, blotted on to nitrocellulose and probed with antibodies raised against affinity purified as well as class specific antibodies of GSTs. These studies revealed higher levels of GST pi protein in breast cancer samples as compared to the corresponding normal tissues. Further studies at mRNA level by RT-PCR confirmed the higher level of expression of GSTP1 and M1. These studies suggest the possible use of GST P1 in the early detection of breast cancers. An interesting observation of the present study is the association of GSTM1 protein with synaptotagmin protein of molecular weight of 42.9kDa in breast cancer samples and not in the normal tissues. This was evident based on Western blot analysis of the proteins from breast cancer tissues with class-specific antibody as well as by MALDI analysis. Further studies on the role of synaptotagmin protein and its functional significance in tissues in association with GSTM1 would be open new windows for better understanding of etiology of breast cancers. Also it can be used as yet another diagnostic marker, after taking up detailed studies.

Summary

The above studies on GSTs, thus have revealed the induction of GST P1 and GSTM1 in most of the breast cancer tissues. Since GSTs are prone for gene deletions, making individuals susceptible for various cancers, further studies were undertaken to determine the frequency of GST gene deletions in breast cancer patients as well as in the age/sex matched healthy volunteers.

These studies revealed higher frequency of GSTM1 gene deletions in breast cancer patients (36%, n=60) compared to those of age and sex matched healthy individuals (18%, n=58). This study suggests a significant positive correlation between GSTM1 gene deletions and occurrence of breast cancer ($P< 0.05$) in the selected South Indian population. The higher incidence of breast cancers in this industrialized/urban canter could be due to increased exposure of the population to environmental pollutants/toxicants, particularly in those with impaired detoxification systems as evidenced by GST gene deletions and point mutations. Further in depth field studies, however, are required to identify the environmental pollutants and urban stress factors that are responsible for higher incidence of breast cancers in this part of South India.

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