IMMUNOHISTOCHEMICAL LOCALIZATION OF GLUTATHIONE S-TRANSFERASES IN NORMAL AND CARCINOMA HUMAN BREAST TISSUE

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

BY

SERPİL OĞUZTÜZÜN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOLOGY

JUNE, 2000

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Tayfur Öztürk
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Gürdal Alaeddinoğlu
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Assoc. Prof. Dr. Serpil Dizbay Sak
Co-Supervisor

Prof. Dr. Mesude İşcan
Supervisor

ABSTRACT

IMMUNOHISTOCHEMICAL LOCALIZATION OF GLUTATHIONE S-TRANSFERASES IN NORMAL AND CARCINOMA HUMAN BREAST TISSUE

Oğuztüzün, Serpil

Ph.D., Department of Biology

Supervisor: Prof. Dr. Mesude İşcan

Co-supervisor: Assoc. Prof. Dr. Serpil Dizbay Sak

June 2000, 177 pages

Epithelial cells, either malignant or normal, generally showed a diffuse cytoplasmic staining and patchy nuclear staining in normal breast and tumour tissue with any of alpha, mu, pi and theta GST antibodies. In addition, there was a great heterogeneity in nuclear staining intensity among normal and tumour cells for the GST isoenzymes. For GST alpha, mu, pi and theta, stronger staining intensity was observed in normal epithelial cells when compared with tumour cells and statistically it was proved that GST expression was dependent on tissue type (normal, in situ, invasive). In normal epithelium there was no difference between the expression of GST isoenzymes. However, in intraductal and invasive tumour tissues, expression was dependent on GST isoenzymes. This study suggested significant relationships between GST alpha expression and patient's age; GST mu, alpha expressions and microcalcification status,

GST theta, alpha expressions and menopausal status; GST pi expression and parity; GST theta expression and hormone therapy. However, there were no significant relationship between GSTs expressions and tumour stage, oestrogen receptor status, chemotherapy status and smoking status. In the western blotting analysis, all samples contained detectable levels of GST pi, mu, alpha, and theta isoenzymes. The levels of alpha and mu class GST proteins varied in normal and tumour tissue significantly among individuals. The mu class GST was expressed in all indivuals. The level of pi class GST protein was found in high concentration but GST theta isoenzyme was expressed at low concentration in all normal and tumour samples. In a previous study using the same cases, 29% of the cytosolic fractions (6 out of 21) had no activity of GST against EPNP which is a substrate for theta class GST. This was most probably associated with the polymorphic expression of the class theta GSTT1-1 where 35-60% of the human population. However, according to the western blotting analysis of the same cases, GST theta protein was present in all of the samples.

Keywords: Breast Cancer, Glutathione S-transferases, Theta, Alpha, Mu and Pi Classes of GSTs, Immunohistochemistry, Western Blotting

ÖZ

NORMAL VE KANSERLİ İNSAN MEME DOKULARINDAKİ GLUTATYON S-TRANSFERAZ İZOZİMLERİNİN İMMÜNOHİSTOKİMYASAL LOKALİZASYONLARI

Oğuztüzün, Serpil
Doktora, Biyoloji Bölümü
Danışman: Prof. Dr. Mesude İşcan
Yardımcı danışman: Doç. Dr. Serpil Dizbay Sak

Haziran 2000, 177 sayfa

Normal ve kanserli meme dokusunda, alfa, mü, pi ve teta GST antikorları ile boyanan tümörlü ya da normal meme epitel hücreleri genellikle yaygın sitoplazmik ve yamasal tarzda çekirdek boyanması göstermiştir. Ayrıca, normal ve tümörlü epitel hücreleri arasında alfa, mü, pi ve teta sınıfı GSTlerin çekirdek boyanma şiddetinde büyük bir heterojenite gözlenmiştir. Normal epitel hücreleri, tümörlü hücrelerle kıyaslandığı zaman GST alfa, mü, pi ve teta için daha kuvvetli boyanma gösterdikleri izlenmiştir, ve GST enzim miktarlarının doku çeşidine (normal, in situ, invasiv) bağlı olduğu istatistiksel olarak da gösterilmiştir. Normal epitelde GST izozimlerinin miktarları arasında bir fark bulunmamıştır. Fakat, in situ ve invasiv tümör dokularında, enzim miktarı GST izozim çeşitine göre değiştiği tespit edilmiştir. Bu çalışma, GST alfa izoziminin miktarı ve

hastanın yaşı; GST mü ve alfa miktarları ve mikrokalsifikasyon durumu; GST teta, alfa miktarları ve menapoz durumu, GST pi miktarı ve parite; GST teta miktarı ve hormon terapi durumu arasında önemli ilişki olduğunu göstermiştir. Fakat, GSTlerin miktarları ve tümör evresi, östrojen reseptör durumu, kemoterapi durumu ve sigara içimi arasında önemli bir ilişki bulunamamıştır. Western blot analizinde, tüm örnekler pi, alfa, mü ve teta sınıfı GSTleri göstermiştir. Normal ve tümörlü dokularda, alfa ve mü sınıfı GST protein düzeyleri bireyler arasında önemli ölçüde değişken olarak bulunmuştur. Bütün örneklerde mü sınıfı GST enzimi olduğu gösterilmiştir. Tüm normal ve tümörlü örneklerde, pi sınıfı GST protein düzeyi yüksek konsantrasyonda, fakat GST teta izozimi düşük konsantrasyonda bulunmuştur. Bölümümüzde daha önce aynı vaka grubunda yapılan aktivite çalışmasında örneklerin %29 unda teta aktivitesi bulunmamıştır. Bu insan populasyonunun %35-60'ında bulunan GST teta polimorfizmini göstermekteydi. Buna karşılık, western blot analizi ile GST teta proteini tüm örneklerde bulunmuştur.

Anahtar kelimeler: Meme Kanseri, Glutatyon S-transferazlar, Teta, Alfa, Mü, ve Pi Sınıfı GSTler, İmmünohistokimya, Western Blot.

TO MY HUSBAND

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors, Prof. Dr. Mesude İşcan and Assoc. Prof. Dr. Serpil Dizbay Sak, for their encouragement, valuable advice and continued guidance throughout this study. Dr. Sak's experience as a pathologist were invaluable in examining the samples and confirming them as being cancerous or normal.

I wish to express my thanks to Prof. Dr. Meral Yücel, Prof. Dr. Tülin Güray and Assoc. Prof. Dr. Esra Erden for their valuable suggestions and corrections.

I am grateful to Dr. E. Juronen for providing the monoclonal GST theta antibody, Prof. Dr. Derek Gadsdon for providing the breast samples of this study, and Assoc. Prof. Dr. Celia Reed for providing support for my experimental study in her laboratory.

My special thanks are due to all of my friends, particularly my lab mates Darren Robinson and Awni Abu Hijleh for their great friendship and support. I am grateful to Prof. Dr. İbrahim Akman for his help in the statistical evaluation of the patient data.

I was supported by British Council as a Chevening Scholar between October 1997 and October 1998 to perform part of my experiments at Liverpool John Moores University School of Biomolecular Sciences. Therefore I am extremely grateful to the British Council for their financial support.

Finally, I would like to express my sincere gratitude to my mother, father, brother, sisters, my cousin Şule, husband and daughter for their great support, patience

and encouragement during the long period of my study. Particularly, my husband Halit was a great support throughout my doctoral studies.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURES

NOMENCLATURE

APES	3-Aminopropyltriethoxysilane
AP	Amplified Alkaline Phosphatase
APS	Ammonium persulfate
A	Acini
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis	N,N'-methylene-bisacrylamide
BSA	Bovine serum albumin
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CDNB	1-Chloro-2,4- dinitrobenzene
CHP, CuOOH	Cumene hydroperoxide
CSS	Cytoplasmic staining score
CYP	Cytochrome P450
D	Ductules
DAB	3,3'-diaminobenzidine tetrahydrochloride
DCIS	Intraduct breast carcinoma (ductal carcinoma in situ)
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEC	Duct epithelial cells
DFS	Disease free survival
DPX	Dibutylthalate in xylene
DTT	Dithiothreitol
EA	Ethacrynic acid
EC	Epithelial cells

EDTA	Ethylenediaminetetraacetic acid
EO	Ethylene oxide
EPNP	1,2-Epoxy-3-(p-nitrophenoxy)propane
ER	Oestrogen receptor
F	Fibroblast
GSH	Glutathione (ϒ-glutamylcysteinylglycine)
GSH-Px	Glutathione peroxidase
GSSG-Rx	Glutathione reductase
GST	Glutathione S-transferases
HE	Haematoxylin Eosin
HRP	Horseradish peroxidase
IDC	Infiltrating duct carcinoma Infiltrating ductal carcinoma
IHC	Immunohistochemistry
L	Lymphocytes
LTC4S	Leukotriene C4 synthase
3-MC	3-methylcholanthrene
N	Normal
NBT	Nitro blue tetrazolium
ND	Normal duct
NDEC	Normal ductal epithelial cells
NGS	Normal goat serum
NSS	Normal swine serum
OS	Overall survival
PAH	Polycyclic aromatic hydrocarbon
PBS	Phophate buffered saline
PMSF	Phenylmethanesulphonyl fluoride
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride membrane
ROS	Reactive oxygen species
RT	Room temperature

S	Stroma
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Se-GPx	Selenium-dependent glutathione peroxidase
StreptABC/HRP	Streptavidin biotin complex/Horseradish peroxidase
T	Tumour
TBS	Tris buffered saline
TC	Tumour cells
TEMED	N, N, N', N'-tetramethylenediamine
Tris base	Acrylamide, hydroxymethyl aminomethane
TSS	Total staining score
TTBS	Tris buffered saline containing Tween-20
VP-16	Etoposide

CHAPTER I

INTRODUCTION

All organisms are exposed continuously to toxic chemicals. The threat provided by such compounds is not a recent problem caused by the activities of the chemical industry, but has existed since life began. Many of the toxic chemicals we encounter are found naturally in the environment. Humans may consume as much as 1.5 g of natural pesticide each day in the form of plant phenols, flavonoids, glucosinolates, and saponins (Ames et al.,1990). All of these naturally occurring pesticides are widespread in food and drink and yield positive results in mutagenicity tests. In addition to these exogenous chemicals, reactive oxygen species (ROS), such as the superoxide radical, hydrogen peroxide, and the hydroxyl radical, which arise as a consequence of aerobic respiration, ionizing irradiation, and inflammation can generate a wide spectrum of harmful carbonyl-containing compounds through interaction with membrane lipids and DNA (Halliwell et al., 1985).

To ensure survival in the face of a wide spectrum of harmful chemicals, various defense mechanisms have evolved to protect cells against noxious compounds. This chemical defence, which is known as detoxification, is carried out principally by the liver. Foreign compounds that enter the body by absorption from the gastrointestinal tract are taken via the portal vein to the liver where they are detoxified. The detoxification products are then excreted in the bile to be voided in the faeces or are taken to the kidneys and excreted in the urine. Kidneys, lung, gastrointestinal tract, and skin are the secondary sites of detoxification.The

metabolism of foreign compounds usually involves two distinct stages, commonly referred to as phases I and II. Phase I metabolism involves an initial oxidation of the xenobiotic by cytochrome P450 (CYP) monooxygenases (Korzekwa et al.,1993). This step is followed by phase II metabolism, which frequently involves conjugation reactions catalyzed by glutathione S-transferases (GST), UDP-glucuronosyl transferases, and sulfotransferases, or reduction reactions catalyzed by the epoxide hydrolase, and quinone reductase. By contrast, protection against ROS and the breakdown products of peroxidized lipid and oxidized DNA is provided by superoxide dismutases, catalase, glutathione peroxidases, GST, aldo-keto reductases, and DNA-repair enzymes.

1.1 Glutathione-S-Transferases

GSTs (EC 2.5.1.18) are a family of multifunctional proteins that function both as important enzymes of detoxification and intracellular binding proteins. Because of this dual function, they have been the research interest of numerous investigators. As enzymes, they catalyze the reaction between the nucleophil reduced glutathione (ϒ-glutamylcysteinylglycine or GSH) and a large number of electrophilic compounds. In addition, they bind a number of amphipathic compounds that they do not metabolize (nonsubstrate ligands) and have been suggested to act as intracellular transport proteins for compounds that have limited solubility in water. GST can also serve as peroxidases and isomerases (Mannervik et al., 1988).

1.1.1 Functions of GSTs

1.1.1.1 Glutathione Conjugation and Detoxification

All GST possess the ability to conjugate GSH with compounds containing an electrophilic center. The electrophilic functional group for conjugation reactions can be provided by a carbon, a nitrogen, or a sulfur atom. The range of compounds that contain electrophilic centers is extremely large and includes the parent

chemical or metabolite of the carcinogens such as aflatoxin B1, benzo[a]pyrene. GST also detoxify the pesticides such as atrazine, dichlorodiphenyltrichloroethane (DDT), the oxidative-damage products such as cholesterol α-oxide, fatty acid hydroperoxides, and the anticancer drugs such as chlorambucil, cyclophosphamide, melphalan, as well as the antibiotic fosfomycin.

The formation of a thioether bond between electrophiles and GSH almost always yields a conjugate that is less reactive than the parental compound (Chasseaud, L.F., 1979), and therefore the actions of GST generally results in detoxification. GSH conjugation is thought to be of value not only because it increases the solubility of hydrophobic xenobiotics and, by preventing their partitioning into membrane lipid, decreases their half-life in the body.

For many toxic xenobiotics, including known carcinogens and products of oxidative stress, glutathione S-conjugate formation represents a detoxification pathway. As shown in Figure 1, the glutathione conjugate is converted to the corresponding cystein conjugate following sequential removal of glutamate and glycine. Cysteine conjugate is either metabolized to a mercapturate by acetylation or cleaved to a mercaptan by beta-lyase (C-S lyase). In addition to the mercapturic acid pathway, methylation of the thiol to form the methylthio-containing metabolite and the glucuronydation of the mercaptan to form the thioglucuronide represent important metabolic steps for the biotransformation of the cysteine conjugate. Thus, excretion of mercapturate and the CH_3S-, CH_3SO-, and CH_3SO_2- containing metabolites are indicative of the *in vivo* formation of glutathione S-conjugates of xenobiotics.

1.1.1.2 Glutathione Conjugation and Toxification

Although the vast majority of GSH conjugates represent detoxification products, several instances exits where GST activity does not result in the detoxification of xenobiotics. For example, 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; these two groups of compounds have been referred to as directly acting toxic GSH conjugates and indirectly acting toxic GSH conjugates.

Directly acting toxic GSH conjugates are formed from a number of alkyldihalides (van Bladeren et al., 1979). Methylene chloride (dichloromethane), a widely used industrial solvent, produces liver and lung cancer in the mouse. It does not, however, cause liver cancer in the hamster or the rat, nor does it induce DNA damage in normal human hepatocytes, suggesting that species-specific differences exist in the GST isoenzyme(s) responsible for the formation S-chloromethylglutathione (Graves et al., 1994; Graves et al., 1995). In humans, different liver cytosol specimens or blood samples display marked interindividual variations in ability to conjugate methylene chloride with GSH, indicating that the enzyme responsible is subject to polymorphic expression (Pemble et al., 1994;Bogaards et al., 1993; Ploemen et al., 1995; Hallier et al., 1993).

1.1.1.3 Peroxidase Activity of GSTs

A significant number of the 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 present nucleophilic attack by GSH on electrophilic oxygen. The substrates that GST reduce include fatty acid, phospholipid, and DNA hydroperoxides. As these compounds are generated by lipid peroxidation and oxidative damage to DNA, it has been proposed that GST, as well as other GSH-dependent enzymes, help combat oxidative stress (Mannervik, B., 1986).

1.1.1.4 Noncatalytic-Binding Activities of GSTs

It has been known for many years that GST are able to bind, both covalently and noncovalently, a wide spectrum of chemicals. Compounds that have been shown to be bound covalently by GST are reactive metabolites formed from carcinogens such as dimethylaminoazobenzene and 3-methylcholanthrene (3-MC)

(Ketterer et al., 1967). It is thought that the covalent binding of these compounds represents a serving to prevent genotoxic electrophiles from interacting with DNA.

All GST bind noncovalently a range of neutral or anionic lipophilic chemicals that are not substrates, including steroid and thyroid hormones, bile acids, bilirubin, "heme", fatty acids, and penicillin (Hayes et al., 1986; Ishigaki et al., 1989; Danger et al., 1992).

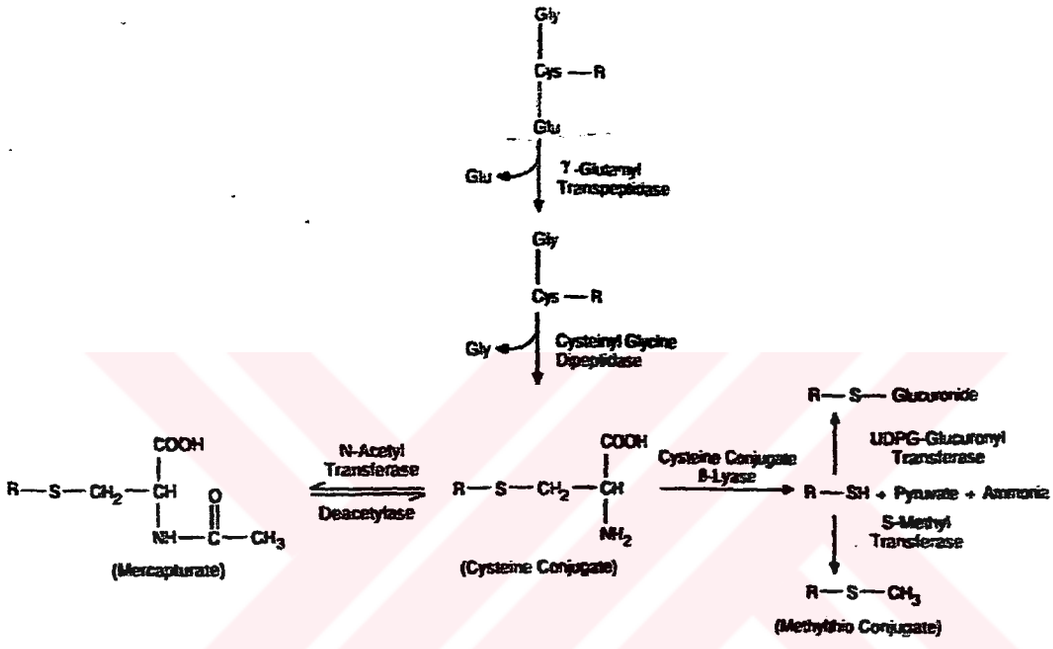


Figure 1. Metabolism of glutathione conjugates (Pickett and Lu, 1989).

1.1.2 Glutathione-S-Transferase Genes

GST are widely distributed in nature, being found in bacteria (Masai et al., 1993), yeast (Tamaki et al., 1990), molds (Saxena et al., 1991), fungi (Sheehan et al., 1993), molluscs (Tomarev et al., 1988), crustacea (Stenersen et al., 1987), worm parasites (Liebau et al., 1994), frogs (Di Ilio et al., 1992), insects (Fournier et al., 1992), plants (Grove et al., 1988), fish (Dominey et al., 1991), birds (Liu et al.,

1991), and mammals (Mannervik et al., 1985). Essentially all eukaryotic species appear to possess multiple isoenzymes.

A large number of cytosolic GST isoenzymes have been purified from rat and human organs and, on the basis of their primary structures, these have been assigned to five separate families designated class alpha, mu, pi, sigma, and theta GST (Buetler et al., 1992; Meyer et al., 1991, 1995).

The hypothesis that these classes represent separate families of GST is supported by the distinct structures of their genes and their chromosomal localizations. The class alpha, mu, pi, and theta GST genes, which have been isolated to date, differ markedly in size and in their intron-exon structures (class sigma GST genes have yet to be characterized). In humans, class alpha, mu, pi, and theta GST genes are located on chromosomes 6, 1, 11, and 22, respectively (Board et al., 1987, 1989, 1992; Ross et al., 1993; Tan et al., 1995).

In addition to the cytosolic GST, at least two membrane-bound GST exist in mammals. These are referred to as microsomal GST (Morgenstern et al., 1983) and leukotriene C4 synthase (LTC4S) (Nicholson et al., 1993). The microsomal GST is involved in the detoxification of xenobiotics, whereas LTC4S, as its name suggests, conjugates leukotriene A4 with GSH.

1.1.3 Nomenclature and Classification of Cytosolic GSTs

An old system for naming the subunits of rat glutathione-S-transferase was based on their relative mobilities in SDS polyacrylamide gel electrophoresis (SDS-PAGE). Originially, subunits Ya, Yb, and Yc, in order of decreasing mobility were distinguished (Bass et al., 1977).

Cytosolic GST in rat liver are dimeric proteins composed of four subunits, such that both homodimers and heterodimers exits, led to the proposed that the nomenclature should reflect the subunit composition (Mannervik and Jenson, 1982).

Each subunits was given a name and an enzyme was named on the basis of its constituent subunits. This principle was adopted by the participants in a workshop on GST in 1983, and it was decided that each variant subunit should receive its own Arabic numeral (Jakoby et al., 1984). A homodimer of subunit 3 should be referred to as GST3-3, whereas a heterodimer of subunits 3 and 4 should be called GST3-4, and so forth. This nomenclature system is of value because it is unambiguous and allows subunit combinations to be simply displayed, but suffers from the disadvantage that it is not immediately obvious to which gene family each subunit belongs.

The nomenclature applied to the rat has not been used for man or other species, making comparison of the different enzymes difficult. In addition, the use of numbers for the various subunits, although being open ended and indicating that these are dimeric proteins, provides little information as to the relatedness of different isoenzymes either within or between species. For this reason, the human GST have been denoted by Greek letters. Five enzymes with basic isoelectric points were referred to as GST α, β, γ, δ and ε (Kamisaka et al., 1975). An enzyme with near-neutral isoelectric point was named GST μ (Warholm et al., 19819 and the acidic protein from erythrocytes (Marcus et al., 1978) or placenta (Guthenberg et al., 1981) GST ρ and π, respectively. The classification of cytosolic GSTs from rat and man, according to this nomenclature system, is shown in Table1.

Recently, a class-based subunit nomenclature system has been proposed. This system groups subunits by gene family and numbers them according to their order of discovery; this system for defining GST was originally devised for the human transferases (Mannervik *et al.*, 1992), but it is generally applicable. In this nomenclature, single capital letter abbreviations are used to signify the **Alpha** (A), the **Mu** (M), the **Pi** (P), the **Sigma** (S) and the **Theta** (T) classes, and Arabic numerals are employed for numbering each of the separate gene products; for example, class **Alpha** subunits are called A1, A2, A3, etc. A single lower case prefix is used to indicate the origin of the enzyme; h, m, r signify human, mouse, and rat, respectively. Hence, the class **Alpha** heterodimer formed between the rat

Ya_1 (A1) and Yc_1 (A3) subunits, rat GST Ya_1Yc_1, is designated rGSTA1-3 (Hayes and Pulford, 1995).

Table 1. Classification of Cytosolic GSTs from Rat and Man According to the Old Nomenclature System (Vos and Bladeren, 1990; Meyer *et al.*, 1991; Hussey and Hayes, 1992).

	Class			
Species	**Alpha**	**Mu**	**Pi**	**Theta**
Rat	1-1 [Ya]	3-3 [Yb_1]	7-7 [Yf]	5-5
	1-2 [YaYc]	3-4 [Yb_1Yb_2]		12-12 (Yrs-Yrs)
	2-2 [Yc]	4-4 [Yb_2]		Yrs-Yrs′
	8-8 [Yk]	3-6		Yrs′-Yrs′
		4-6		
		6-6 [Yn]		
Man	α, β, γ [B_2B_2]	μ	π	θ (T1-1)
	δ [B_1B_2]			θ (T2-2)
	ε [B_1B_1]			

GSTs are officially only admitted to the class-based subunit nomenclature once their primary structures have been determined. A preliminary class-number may be given, designated by an asterisk, to a "new" subunit once there is sufficient evidence to indicate that the novel subunit is genetically distinct from previously described polypeptides (e.g., rGSTM3-5*). Classification of cytosolic GSTs from rat and man according to this nomenclature system is shown in Table2.

Table 2. Classification of cytosolic GSTs from rat and man according to the new nomenclature system (Hayes and Pulford, 1995).

Species	Class			
	Alpha	**Mu**	**Pi**	**Theta**
Rat	RGSTA1-2 [Ya_1Ya_2]	rGSTM1-1 [Yb_1Yb_1]	RGSTP1-1 [YfYf]	rGSTT1-1 [GST 5-5]
	RGSTA1-3 [Ya_1Yc_1]	rGSTM1-2 [Yb_1Yb_2]		rGSTT2-2 [Yrs-Yrs] [12-12]
	RGSTA2-3 [Ya_2Yc_1]	rGSTM2-2 [Yb_2Yb_2]		rGSTT2-2′ [Yrs-Yrs′]
	RGSTA3-3 [Yc_1Yc_1]	rGSTM1-3 [Yb_1Yb_3]		rGSTT2′-2′ [Yrs′-Yrs′]
	RGSTA4-4 [YkYk]	rGSTM2-3 [Yb_2Yb_3]		
	RGSTA1-5 [Ya_1Yc_2]	rGSTM3-3 [Yb_3Yb_3]		
	RGSTA2-5 [Ya_2Yc_2]	rGSTM4-4 [Yb_4Yb_4]		
	RGSTA3-5 [Yc_1Yc_2]	rGSTM3-5* [Yb_3Yn_2]		
	RGSTA2-2 [Ya_2Ya_2]	rGSTM6*-6* [YoYo]		
Man	HGSTA1-1 [ε] [B_1B_1]	hGSTM1a-1a	HGSTP1-1 [π]	hGSTT1-1
	HGSTA1-2 [δ] [B_1B_2]	hGSTM1a-1b		hGSTT2-2
	HGSTA2-2 [α, β, λ]	hGSTM1b-1b		
	HGSTA3-3*	hGSTM1b-2		
	HGSTA4-4*	hGSTM2-2		
		hGSTM2-3		
		hGSTM3-3		
		hGSTM4-4		
		hGSTM5-5		

1.1.4 Substrates

1.1.4.1 Substrates for Characterization of Enzymes

Many of the substrates are reactive compounds that could react with nucleophilic chemical groups in proteins and nucleic acids and thus cause toxic effects, mutations and cancer. The nucleophilic attack catalyzed by the glutathione transferases was considered to be targeted only at an electrophilic carbon atom. Later, it was established that electrophilic nitrogen in nitrate esters, sulfur in organic thiocyanates or disulfides, and oxygen in organic hydroperoxides could serve as alternative targets in the catalyzed reactions.

Most substrates used are products of modern chemical industry and have no biological relevance. Furthermore, most of these compounds give comparatively low enzyme activities. Nevertheless, some of these substrates are valuable tools for the characterization and identification of the different forms of GSTs. The single most important substrate used for the demonstration of multiple forms of GSTs in various biological species is 1-Chloro-2,4- dinitrobenzene (CDNB). This compound was originally used as a substrate for the "aryltransferase", but was also recognized as a "general substrate" for the GST (Clark *et al.*, 1973).

Notwithstanding CDNB significance for detection of GST activity, it should be stressed that certain forms of the enzyme express low activity with this substrate. Rat GST 5-5 (Meyer *et al.*, 1984) is an example of enzyme that display low activity with this substrate. Obviously, several substrates should be used in the screening of new sources of the enzymes.

In some biological species, certain forms of GSTs have been clearly distinguished by their different activities with a given substrate. In another study of some animal GSTs, it was similarly found that, although different enzyme forms in the same tissue appeared to show a degree of "cross-specificity" for different substrates, their activity profiles were clearly distinct (Clark *et. al.*,1973).

Class Alpha GSTs are highly active with cumene hydroperoxide (CHP, CuOOH) (Mannervik,1985). Class Mu transferases have been noted to be highly active with epoxides (Warholm *et al.*, 1983; Mannervik, 1987) eg. trans-stilbeneoxide.(Seidegard *et al.*, 1986)

Class Pi transferases display comparatively high activity with ethacrynic acid (EA) but they can be distinguished better by 4-hydroxyalkelans as substrates (Danielson *et al.*,1987).

In contrast to the members of the other classes, the theta class isoenzymes have little activity with the model substrate CDNB and do not bind significantly to immobilized glutathione or hexylglutathione affinity matrices. Up to now, two distinct theta class GSTs, namely GST T1 and GST T2 have been isolated from human liver. These isoenzymes have significantly different substrate specificities. GST T1 with activity, amongst other substrates, towards 1,2-Epoxy-3-(p-nitrophenoxy)propane (EPNP) and dichloromethane (DCM), and GST T2 specifically lacking activity towards EPNP, but metabolizing reactive sulfate esters.

Within a class, different isoenzymes in the same biological species may be further distinguished by the use of additional substrates.

Even if the above-noted-class-distinguishing substrates have been useful, they do not give a definitive distinction between transferases of different classes. A single substrate, in most cases, gives specific activities for members of a given class, which fall in a range that partly overlaps the range of the values of another class.

1.1.4.2 Endogenous Substrates

Substrates for GSTs do not occur naturally and have no significance in relation to the true biological function of the enzymes, even if some of them may be

relevant to toxicology. However, it has been stressed that oxidative metabolism of a variety of endogenous substances gives rise to reactive electrophiles that should be considered possible natural substrates (Mannervik, 1986; 1987). In addition an organism is also exposed to a variety of potentially toxic natural compounds which are produced by other species in the biosphere (Ames, 1983).

Glutathione-dependent enzymes, including the transferases, evolved in aerobic organisms in response to the requirements of inactivation of toxic products of oxygen metabolism (Mannervik, 1986).

Quinones represent one class of reactive compounds which may be detoxified by glutathione conjugation. A possible endogenous substrate is dopaquinone, which has been shown to be conjugated with glutathione in human malignant melanoma (Agrup *et al.*, 1977). This tumor has been found to contain a high concentration of class Pi-GSTs (Mannervik *et al.*, 1987).

Organic hydroperoxides, but not the less hydrophobic H_2O_2, are substrates for glutathione transferases. In the case of peroxidized DNA, the rat GSTs, especially transferase 4-4, were shown to have the highest activities (Mannervik, 1985). With hydroperoxides previously used, class Alpha enzymes have been found to show the highest values.

Epoxides is a third group of substrates that can be formed by oxidation of carbon-carbon double bonds in vivo. Epoxides may be derivatives of naturally occurring compounds as well as of xenobiotics and are known as mutagenic and carcinogenic substances. Rat glutathione transferase 5-5 was the first pure enzyme shown to have significant activity with epoxides.

Arachidonic acid is an important polyunsaturated fatty acid that gives rise to several epoxide derivatives. Arachidonic acid oxides as well as leukotriene A and its non physiological methyl ester have been shown to serve as substrates for the well-characterized rat and human glutathione transferases. Cholesterol alpha-

epoxide is another naturally occurring substrate for the GSTs (Meyer and Ketterer, 1982).

Alkenes are produced during lipid peroxidation. 4-hydroxyalkenals have been shown to be excellent substrates for GSTs (Alin *et al.*, 1985; Ishikawa *et al.*, 1986).

These examples lead to the conclusion that several types of toxic electrophiles which are produced intracellularly may function as "natural" substrates for the GSTs. The variety of functional groups and the carbon skeletons to which they are attached may be one of the causes why so many of GSTs have evolved.

1.1.4.3 Substrates Related to Cancer and Drug Resistance

GST contribute to the detoxification of a number of potentially harmful chemicals that we encounter daily, either in the air we breathe, the food we eat, or the medication we receive. Table 3 lists some of the compounds detoxified by GST.

Table 3. GSTs Substrates from Different Compound Categories (Hayes and Pulford, 1995).

Compound Type	**Substrates**
Metabolites of carcinogens/ mutagens	Epoxide (e.g. benzo(a)pyrene-7,8-diol-9,10-oxide) Aflatoxin B1 exo-8,9-epoxide Polycyclicaromatichydrocarbons (PAH) Sulfate esters
Environmetal pollutants and Pesticides	Cumene hydroperoxide Atrazine Dichlorodiphenyltrichloroethane (DDT)
Oxidative-damage products	Fatty acid hydroperoxides Cholesterol α-oxide Acrolin
Toxic chemicals	Ethylene oxide Methyl bromide Methyl chloride Styrene oxide Trans-Stilbene oxide
Antibiotics	Fosfomycin Ampicilin Penicillin
Anticancer drugs	Nitrogen mustard Chlorambucil Mechlorethamine Melphalan Cyclophosphamide Bleomycin Adriamycin

Many chemical carcinogens are electrophilic compounds that may be inactivated by reaction with glutathione (Chasseaud, 1979). Such detoxification reactions may inhibit binding of carcinogens to DNA and thereby prevent initiation of cancer. It is also possible that compounds acting as tumor promoters are inactivated by reactions involving glutathione.

Epoxides represent an important group of mutagenic and carcinogenic compounds which illustrate important aspects of GSTs in detoxication. In general, class Mu enzymes are the transferases that show the highest activities with most

epoxides (Mannervik, *et al.*,1987). It has been proposed that transferase mu may have an especially important role in the detoxification of geno- and cytotoxic epoxides (Warholm *et al.*, 1983). Particularly noteworthy is the discovery that only 60% of the human population express transferase mu (Warholm *et al.*, 1980), and a survey of smokers demonstrated that individuals lacking transferase mu had a significantly higher incidence of lung cancer than those who display transferase mu activity (trans-stilbene oxide activity) (Seidegard *et al.*, 1986).

Polycyclic aromatic hydrocarbon (PAH) represent a major group of chemical carcinogens, first identified in coal tar and associated with several occupational cancers. These compounds are commonly encountered in combustion products such as car exhaust fumes, cigarette smoke,and coal soot. PAH include the compounds benzo [a]pyrene, benz[a]anthracene, 7-methyl-benz[a]anthracene, 7,12-dimethylbenz[a]anthracene, and 3-methylcholanthrene. Most PAH require activation by CYP isoenzymes before they are able to exert their harmful effects. The ultimate carcinogens of PAH are epoxide-containing metabolites, many of which are substrates for class mu and pi GST (Table 2). The ultimate carcinogens of methylchrysene are sulfate esters, formed by the combined actions of CYP and sulfotransferase isoenzymes. These reactive sulfate esters are metabolized by class theta T2 subunits (Hiratsuka et al., 1990).

It might be expected that the mammalian class theta GST enzymes, which are more similar to the GST in invertebrates than the mammalian class alpha, mu, or pi enzymes, would be most active class of mammalian enzyme toward many of the insecticides and herbicides that are metabolized by plant and insect GST enzymes.

GST T1 has been shown to be a major enzyme in the metabolism of mono- and dihalomethanes in human erythrocytes (Hallier et al. 1990; Thier et al. 1991). Other substrates of GST T1 are ethylene oxide (Foest et al. 1991) monoepoxybutene and diepoxybutane (Guesgerich et al. 1995; Wiencke et al. 1995). Many of these substrates are known or suspected carcinogens. The GST T1 gene deletion may therefore modify the individual risk associated with exposure to

toxic and carcinogenic chemicals. GST T2 has significant activity with a cumene hydroperoxide and 1-menapthyl sulfate. The activity of GST T2 with a range of secondary lipid peroxidation products such as the trans, trans-alka-2,4-dienals and trans-alk-2-enals, as well as its glutathione peroxidase activity with organic hydroperoxides suggest that it may play a significant role in protection against the products of lipid peroxidation.

In the discussion of the relationship of glutathione conjugation of DNA binding, mutagenesis, and cancer, it also should be noted that some chemicals are activated by the conjugation reaction. vis-Dihaloalkenes are such compounds that have been shown to give mutagenic glutathione derivatives in the presence of GSTs (Igwe, 1986). A general protective effect of glutathione conjugation is not always true.

The transferases that are primarily involved in activation reactions include microsomal and class theta GST. In view of the possibility that class alpha, mu, and pi GST sequester GSH conjugates, it would be interesting to know whether the presence of these enzymes can modulate the toxicity of compounds that are activated by conjugation. For example, methylene chloride is a hepatocarcinogen in mice but not rats (Graves et al., 1994 and 1995). It is not known whether this selective toxicity is due to the high activity of murine class theta GST for methylene chloride or failure of mouse liver to express constitutively the mGSTA1 and A2 subunits.

Several antibiotics are bound by GST but, to date, only one enzyme, a GST from *Serratia marcescens*, has been reported to conjugate GSH with the epoxide-containing antibiotic fosfomycin (Arca et al., 1990). Mammalian class theta T1 subunits have a preference for small epoxide-containing compounds, such as 1,2-epoxybutane, 1,2-epoxypropane, rather than the relatively large PAH-epoxides. It is therefore possible that rodent and human GSTT1-1 can metabolize fosfomycin.

Another undesired effect of glutathione-dependent reactions appears to be the inactivation of drugs used in cancer chemotherapy. Several lines of evidence suggest that glutathione and glutathione transferases are involved in drug resistance. The cellular resistance acquired by extended drug exposure has in many cases been found to be associated with elevated levels of GST, especially the class Pi enzyme (Deffie *et al.*, 1988; Batist *et al.*, 1986; Teicher *et al.*, 1987). Little is known about the specificities of the different types of GST for chemotherapeutic compounds.

The ability of GST to detoxify anticancer drugs has attracted considerable interest. Class mu and microsomal GST catalyze the denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Berhane et al., 1993), whereas class alpha GST catalyze the conjugation of GSH with the nitrogen mustards chlorambucil, mechlorethamine, and melphalan. Human GST are active toward the aldophosphamide and aziridinium metabolites of cyclophosphamide. Several other alkylating agents are used in cancer chemotherapy, such as triethylenemelamine and busulphan, that may also serve as GST substrates. Anticancer drugs, such as adriamycin and bleomycin, can give rise to ROS through redoxcycling, or, in the case of cyclophosphamide, may be metabolized to reactive carbonyl-containing compounds. GST, by their ability to reduce organic hydroperoxides or conjugate α,β-unsaturated aldehydes, may be involved in protecting against the cytotoxicity of adriamycin, bleomycin, and cyclophosphamide. Class pi GST have the highest activity toward the α,β-unsaturated aldehydes acrolein and base propenals. The microsomal and class alpha GST exhibit the highest peroxidase activities.

1.1.5. Tissue Distribution of GSTs

The tissue distribution of GSTs has received considerable attention over the past few years and GST isoenzymes are shown to demonstrate a large variability in tissue distribution.

1.1.5.1 Rat Tissues

In the rat, each subunit seems to be present in almost every organ, however, the level of expression differs significantly from tissue to tissue (Vos and Bladeren, 1990). The highest amount of total GST protein is present in the liver (3-5% of total soluble protein) (Hayes and Mantle, 1986b).

Besides the large variability in tissue distribution, GST isoenzymes may also demonstrate a differential localization within a certain tissue or even within cells. Thus, in liver, highest activity and immunohistochemically stainable protein with antibodies raised against isozymes 1-2, 3-4, and 5-5, have been reported in the centrolobular region (Kera *et al.*, 1987; Redick *et al.*, 1982). Anti 3-4 and anti 5-5 have been found to produce intense staining within the bile duct epithelium, whereas 1-2 is not apparent within the bile duct. Isozyme 5-5 is present in parenchyma cell nuclei. Anti 1-2 and anti 3-4 on the other hand have caused perinuclear staining (Redick *et al.*, 1982).

1.1.5.2 Human Tissues

The cytosolic mammalian GSTs have been divided into four species independent classes: alpha, mu, pi, and theta (Mannervik, 1985; Meyer *et. al.*, 1991; Hussey and Hayes, 1992). Wide interorgan and interindividual variation of these four classes has been reported in different tissues (Sherman *et. al.*, 1983; Carrigall *et. al.*, 1988) (Table 4).

In liver, antibody to alpha GST gave strong staining of the cytoplasm and nuclei of all morphologically normal hepatocytes and antibody to the pi GST left the hepatocytes unstained but stained the bile duct structures in all twenty-nine biopsy specimens within the portal tracts and sometimes within the lobule, suggesting that ducts of Hering were being stained. Thirteen of the 29 biopsy tissues showed weak to moderate hepatocyte cytoplasmic staining with the antibody to mu GST (Champbell *et. al.*, 1991; Hiley *et. al.*, 1988). Immunoblot analysis of

various tissue cytosols suggested that like to other polymorphic GST mu, GST theta class is widely expressed in different human tissues. GST T1 is present in lung, kidney, brain, skeletal muscle, heart, small intestine and spleen, but not in lymphocytes (Juronen et al. 1996). Using the rat GSTT2 probe and antibody, the levels of this enzyme in human liver were very low and showed a general distribution throughout the lobule. The antibody did however reveal high concentrations of this enzyme in the bile ducts of human liver (Green et al., 1996).

In kidney, the antibody to the alpha GST stained the proximal convoluted tubules. In contrast, antibody to the pi-GST stained collecting tubules and calyceal epithelium strongly. This staining appeared to be concentrated within the distal convoluted tubule rather than the proximal convoluted tubule. Antibody to the mu-GST yielded weak results in the 18 renal sections studied.(Campbell *et. al.*, 1991).

Table 4. Tissue Distribution of Human GST Subunits by Immunohistochemical Staining.

Tissues	GST α	GST π	GST μ	GST θ	References
Liver	Strong	Weak	Weak	Weak	Campbell et al., 1991 Green et al. 1996
Kidney	Strong	Strong	Weak		Campbell et al., 1991
Adrenal Gland	Strong	Weak	Weak		Campbell et al., 1991
Testis	Strong	Strong	Weak		Campbell et al., 1991
Ovary	Strong	Moderate	Weak		Campbell et al., 1991
Placenta	no staining	Strong	No staining		Campbell et al., 1991
Thyroid	no staining	Strong	No staining		Campbell et al., 1991
Small intestine	Weak	Strong	Strong		Hayes et al., 1989
Stomach	Strong	Weak	No staining		Campbell et al., 1991
Gastric	Strong	Strong	Weak		Peters et al., 1990
Esophagus	no staining	Strong	No staining		Peters et al., 1993
Colon	Strong	Strong	Weak		Ranganathan et al., 1991
Pancreas	Strong	Strong	Strong		Hayes et al., 1990
Salivary gland	Weak	Strong	No staining		Campbell et al., 1991
Skin	Weak	Strong	Moderate		Campbell et al., 1991
Brain	no staining	Moderate	Moderate		Campbell et al., 1991
Spleen	no staining	Weak	No staining		Campbell et al., 1991
Breast	Weak	Strong	Strong		Forrester et al., 1990
Lung	Strong	Strong	Weak	Weak	Anttila et. al.,1993 Green et al. 1996
Urinary Bladder	Strong	Strong	Strong		Singh et al., 1991

The antibody to alpha-GST stained the luteinized cells strongly in ovary. With the antibody to mu-GST all five cases gave negative results in follicular structures with variable weak reactions in the stroma and vessels. Antibody to the pi-GST yielded a moderate reaction in the luteinized cells and in the primordial follicles (Campbell *et. al.*, 1991).

Pi and mu-GST stained strongly in the small intestinal epithelium in the cells lining both the villi and crypts. Staining for alpha was found in the cells lining the villi but not crypts whilst microsomal GST was found only in the cells lining the crypts (Hayes *et. al.*, 1989).

In the five stomach examined, the oxyntic cells stained strongly with the antibody to alpha-GST and negatively with that to the mu-GST and pi-GST. The antibody to the pi-GST tended to concentrate in the mucous secretory cells (Campbell *et al.*, 1991).

Immunoreactivity for alpha and pi-GST was present in ten of thirteen samples (77%), with GST-mu expressed in six (46%)(McKay *et al.*, 1993). The columnar and crypt epithelial cells had the highest levels of GST isoenzymes (GST-pi), perhaps reflecting both their proximity to the intestinaltract contents and their 'first barrier' physiological function. Interestingly, endocrine cells were negative for expression (Ranganathan *et al.*, 1991)

In two postmortem cases, the antibody to alpha-GST failed to stain any structures in brain whereas both the mu and pi antibodies gave moderate reactions with glial cells and small vessels. The neurons remained unstained with all three antibodies in these two cases (Campbell *et. al.*, 1991).

All three classes of GST were expessed in breast tissue. In the majority of samples, GST-pi content was present and GST-mu is known to be subjected to genetic polymorphism and is not expressed in 40-50% of the population. The level of GST-mu (when expressed) was subjected to large individual variation (Forrester *et. al.*, 1990).

Alpha and pi-GST were found to be the most abundant GSTs in human lung, being present in the bronchial and bronchiolar epithelium of all individuals studied. Mu-GST, a polymorphic mu-class enzyme, was detected in lung tissue at

very low levels. The immunostaining for GSTs in general was most intense in the bronchial epithelium decreasing in the distal airways, in contrast to the peripheral localization of the polycyclic aromatic hydrocarbons activating the P450 enzymes (Anttila *et. al.*, 1993). In human lung, the rGSTT2 antibody showed low levels of protein in the bronchiolar epithelium. GSTT1, on the other hand, appeared to be completely absent from the large bronchioles and was only found in a few Clara cells and ciliated cells at the alveolar/bronchiolar junction (Green *et al.* 1996).

The reason for the extensive differences noticed for GSTs isozyme patterns in different tissues is not clear, but the strikingly different distribution of the pi, alpha, mu and theta GST in the liver, pancreas, kidney, placenta, breast, colon and other tissues may help to shed new light on the functions of these isoenzymes and may allow additional insight into the interindividual and interorgan differences in susceptibility to tissue damage and carcinogenesis after exposure to certain xenobiotics. This is possibly also the case for the developmental patterns observed in several tissues.

1.1.6 Regulation of GSTs During Carcinogenesis in Humans

GST isoenzymes show large differences between different tumors. There is considerable interest in the association between pi class GST and malignancy after the discovery that increased expression of this enzyme occurs in many tumors (Howie *et al.*, 1990; Kodate et al,. 1986; Di llio *et al.*, 1988; Eimoto *et al.*, 1988; Lewis *et al.*, 1989; Moscow *et al.*, 1989; Campbell *et al.*, 1991; Shea *et al.* , 1988) (Table 5).

Table 5. Altered GST enzymes in various tumor types by Immunohistochemical Staining.

Tumor type	**Decreased isoenzyme expression**	**Increased isoenzyme expression**	**References**
Breast	GST alpha	GSTpi, mu	Cairns, 1992
Colon	GST mu	GST alpha	McKay, 1993
Lung	--	GST alpha, pi	Di Iiio, 1988 Howie, 1990
Gastric	GSTmu	GST pi	Peters, 1990
Kidney	GSTalpha,mu	--	Di Iiio, 1987
Liver	GST alpha	--	Howie, 1990
Uterine cervix	--	GST pi	Riou, 1991
Stomach	GSTalpha,mu	GST pi	Howie, 1990

The class pi enzyme is not consistently overexpressed in human hepatoma, and is expressed in hepatocytes of patients with alcoholic liver disease, which has prevented its use as a histochemical marker for liver cancer. Human GSTP1-1 has been reported to be overexpressed in a variety of malignancies including carcinoma of the gastric, lung, kidney, ovary, pancreas, esophagus, and stomach (Harrison et al., 1989; Shiratori et al., 1987; Di Ilio et al., 1987 and 1988; Eimoto et al., 1988; Shea et al., 1988; Moscow et al., 1989;Peters et al., 1993; Howie et al., 1990). The class pi enzyme is also overexpressed in breast cancer, where levels of this enzyme are inversely related to estrogen-receptor levels in the tumor (Moscow et al., 1988; Howie et al., 1989; Cairns et al., 1992).

Measurement of the levels of hGSTP1 protein within tumor samples may be of clinical value in certain malignancies. Gilbert et al. (1993) reported that high hGSTP1 levels in breast cancer is a significant predictor of early recurrence of disease in patients who were treated surgically without chemotherapy. Similarly, Grignon et al. (1994) proposed that the expression of class pi GST may be of prognostic value in patients with renal cell carcinoma, and Mulder et al. (1995)

suggested high hGSTP1 levels represent a poor prognostic index in patients with colorectal cancer. In ovarian tumors and acute nonlymphoblastic leukemia, low hGSTP1 expression has been reported to be associated with responsiveness to chemotherapy (Green et al., 1993; Hamada et al., 1994). In contrast, other investigations have failed to find a correlation between GST levels and response to chemotherapy (Okuyama et al., 1994; Murphy et al., 1992), and it is clear that further clinical studies are required to establish the prognostic value of GST measurements.

Measurement of plasma hGSTP1-1 levels has shown that this protein is dramatically increased in lung cancer (Beckett et al., 1993), but the extent of expression of this protein in many tumors is frequently insufficient to allow the enzyme to serve as a general tumor marker.

Although increased class pi GST levels have been found in many human cancers, it is clearly not involved in all carcinomas. An immunohistochemical study of hGSTP1-1 failed to detect the class pi subunit in 88 of 91 prostatic cancers (Lee et al., 1994) and Colon (McKay et al., 1993).

It is important to note that GST pi was not the only GST subunit expressed in high levels and that the expression of other subunits made a significant contribution to overall GST content (Table 3). There is little information on the expression of extra-hepatic alpha and mu class GST in normal and tumor tissues in man. In human kidney, stomach and liver high levels of alpha class and significant levels of mu class subunits were expressed. In addition, high levels of mu class GST has been measured in breast tissue.

In tumors derived from kidney, stomach (all of which express high levels of alpha GST in normal tissue) a very marked reduction in the expression of alpha and the mu class GST has been observed.

Little information on human GST theta class is available yet. In addition, there are few reports for the GST theta distribution on human cancer tissue.

In conclusion, many GST subunits, from distinct gene families contribute to tumor GST levels. Both increased and reduced levels of GST expression in tumors was observed. It remains to be established whether these differences in expression of alpha, mu, pi and theta subunits are important in determining sensitivity to cytotoxic compounds.

1.1.7 GST Isoenzymes in Human Breast Cancer Tissues

İşcan, M. and colleagues (1998) reported that the GST activity toward CDNB and ethacrynic acid (EA), and selenium-dependent glutathione peroxidase (Se-GPx) activities and GSH levels in breast tumours were significantly higher than those of tumour free tissues. Di Ilio and co-workers (1985) reported that when comparison was made between normal and neoplastic tissues of the same individual, GST and glutathione reductase (GSSG-Rx) and glutathione peroxidase (GSH-Px) activities were higher in all tumour cases.

Perry and co-workers (1993) found that GSH levels in primary breast tumours were more than twice the levels found in normal breast tissue, and levels in lymph node metastases were more than four times the levels in normal breast tissue. No correlation was found between tumour GSH levels and common clinical parameters such as tumour size, nodal status, stage, oestrogen receptor levels, or progesterone receptor levels. It has been concluded that GSH appears to be a marker of breast malignancy that is independent of hormonal receptor status and stage and may be a marker of cells with increased potential for dissemination.

Forrester and co-workers (1990) reported that all three classes of GST were expressed in breast tissue. The pi and mu-class enzymes preponderate. Levels of the alpha class GST were very low.The levels of GST-pi were relatively high in almost all the breast samples and were subject to some individual variation with

occasional samples containing high levels of this protein. In the majority of samples, GST-pi and GST-mu content was similar both between the tumours and between normal and tumour tissue from the same patient. These findings are consistent with those of Moscow and colleagues (1988) who measured GST-pi mRNA levels in breast tissue.

Kelley and colleagues (1994) have found that in infiltrating ductal carcinoma tissue, both the levels and the variability of the GST isoenzymes are elevated relative to normal breast tissue. GST-pi, the predominant isoenzyme in normal breast tissue, was elevated in matched cancer tissue of the majority of patients. Their findings of GSTM1 in the tissues of 40% of patients, are consistent with the frequency of the M1-nulled phenotype in the general population, The majority of patients studied showed an absence of known alpha-class GSTs in normal or breast cancer, although a small number of patients did display low levels of GSTA1.

Breast tissue cells (either malignant or non-malignant) staining with any of the three GST antibodies (GST-pi, GST-alpha and GST-mu) generally showed both nuclear and cytoplasmic positivity. Patterns of expression within breast tissues were distinct for each GST isoenzyme (Cairns et al.,1992). Cairns et al. reported that GST-pi in 47% of 74 breast cancers, was detected in the neoplastic epithelium, in many tumours, expression was far greater in the accompanying stromal cells and inflammatory infiltrate, and normal mammary epithelium (including the myoepithelial layer) stained consistently and strongly. Approximately 50% of the population (those of the null phenotype) do not express GST-mu. There was a strong correlation between GST-mu expression by normal epithelium and by tumour, and 42% of the tumours showed at least focal positivity. Only 19% of our cases showed evidence of GST-alpha expression by tumour epithelium and there was little staining of non-neoplastic tissues. Howie et al.,(1989) detected GST-alpha in all 68 breast tumours that they studied, but median concentrations for the B1 and B2 subunits were over 25 times smaller than those for the pi and mu isoenzymes.

Forrester and co-workers (1990) has been reported that the polymorphic mu-class GST was expressed in ~50% of the samples which is consistent with the frequency of this polymorphism in the population suggests that its absence does not confer a greater risk for this tumour type. It has been reported that the polymorphic GST mu isoenzyme is a genetic determinant of susceptibility to lung cancer (Seidegard et al., 1986). This is consistent with the findings of Kelley and co-workers (1994). Zhong et al.,(1993) and Ambrosone et al.,(1995) suggested, however, that GSTM1 may confer increased susceptibility to breast cancer at an earlier age. It was found that among individuals diagnosed at an earlier age, there was a preponderance of those with the null genotype. Therefore, GSTM1 genotype were suggestive of an elevated risk among the youngest postmenopousal women. Cigarette smoking did not affect the association between GSTM1 and breast cancer risk but did appear to modify risk associated with the CYP1A1 polymorphism.

Bellamy and co-workers (1994) analysed GST-pi expression by immunohistochemistry of a series of 92 patients with intraduct breast carcinoma (ductal carcinoma in situ, DCIS). This study of a large number of patients has demonstrated loss of GST-pi expression in DCIS when compared with normal breast epithelium. These results differ from those in other epithelia in which GST-pi expression is increased in dysplasia and in carcinoma compared with normal cells (Sato et al., 1989; Howie et al.,1990). The results also indicated that loss of GST expression can occur at a relatively early (i.e. intraepithelial) stage in breast carcinogenesis, but that this loss is not an irreversible event, as evidenced by altered GST-pi status in some recurrences. Furthermore, both GST-positive and GST-negative DCIS patients developed invasive carcinoma in this study. Hence, GST-pi has not been found to be a marker for tumour progression in DCIS. Their results indicated that assessment of GST-pi staining in non-invasive breast carcinoma did not have a clinical utility.

GST-pi has been proposed as a tumour marker in some human tumours (Soma et al., 1986; Di Ilio et al., 1987 and 1988). However, this is not the case in breast cancer. In some families a slight elevation in the level of GST-pi expression

was seen, but this was not a general phenomenon. Immunohistochemical studies showed that normal breast epithelial cells as well as infiltrating plasma cells all expressed GST-pi (Forrester et al.,1990).

Glutathione S-transferase theta was firstly purified and characterized from normal and cancerous human breast tissue (Abu-Hijleh, 1999).

Several reports had shown that GST-pi expression is inversely correlated with oestrogen receptor (ER) levels in primary breast cancer. It has been reported that ER and progesterone receptor (PR) negative breast tumours have significantly higher levels of GST-pi mRNA and protein levels than ER and PR positive tumours (Moscow et al., 1988; Howie et al.,1989; Gilbert et al.,1993). The reason for this inverse expression of GST-pi and hormone receptor is not clear. Forrester et al.,(1990) suggested that tumour GST-pi concentration did not appear to be directly regulated by the ER. It may be that the level of differentiation is an important determinant in the level of tumour GST-pi expression. Also, they reported that there was no association between the level of expression of either mu class GST protein with ER status. Other studies suggested that there is no connection between the total GST or GST-pi activity and ER or PR status (Clapper et al.,1991), or is much weaker than previously reported (Shea et al.,1990; Colovai et al., 1992).

Gilbert and co-workers (1993) demonstrated that increased GST-pi expression was associated with a poorer disease free survival (DFS) and overall survival(OS) in node-negative breast cancer patients. The effect of increased GST-pi expression on prognosis was independent of ER and PR status, tumour size, nuclear grade, and patient age. These results indicated that GST-pi expression is a potentially useful marker for identifying node-negative patients at significant risk for relapse and death who might benefit from adjuvant chemotherapy.

The values of GST activity were also compared with clinical and histological findings of the malignant breast tumours. Colovai and co-workers (1992) found significant correlation between GST activity and axillary node status,

which is an important marker of prognosis. Higher values of GST activity correspond to the tumours with one or more involved nodes, so the higher levels seem to be connected to a poorer prognosis.

No relationship has been observed between GST expression and age in breast cancer (Moscow et al.,1988). Clapper and colleagues (1991) indicated that GST activity levels were significantly higher in stage I-II tumours than in stage III-IV tumours. They also failed to define a relationship between either age or sex and the GST activity of breast tissue. Similarly, Howie and co-workers (1989) reported that no relationship could be found between GST expression and age, menopausal status, lymph node involvement or tumour stage.

1.1.8 Contribution of GSTs to Drug Resistance

Chemotheraphy plays a major role in the treatment of malignant diseases. The development of resistance to chemotherapeutic agents, at concentrations which were once effective for treatment, is a major obstacle in the clinical treatment of cancers. This resistance can be either intrinsic to the tumor or acquired due to non-lethal drug exposure and is often observed with many drugs complicating attempts to treat cancer. In a variety of experimental models, increased tolerance of toxic xenobiotics and antineoplastic drugs is associated with increased expression of GST (Tew, 1996).

The recognition that GST levels are frequently elevated in cell lines selected for resistance to anti-cancer drugs has served as a stimulus to determine the role of GST in protection against chemotherapeutic agents. Many groups have found that GST are overexpressed in a variety of human and rodent cell lines that were selected for resistance in vitro to adriamycin, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), chlorambucil, cyclophosphamide, etoposide (VP-16), melphalan, and vincristine. In addition to these cell lines with acquired drug resistance, comparison among related breast, bladder, or colon cell lines, which display different intrinsic levels of resistance toward hepsulfam, mitomycin C, or

mitoxantrone, has revealed a correlation between GST expression and the level of drug resistance. As table 6 shows, the majority of these resistant lines contain elevated levels of class pi GST. However, many of the cells that are resistant to nitrogen mustards contain increased levels of class alpha GST (Schecter et al., 1991; Robson et al., 1987; Yang et al., 1992; Wareing et al., 1993).

Table 6. Altered GST Expression in Drug-Resistant Human Breast Cancer Cell Lines (Hayes et al., 1995)

Drug	Fold Resistance	Cell Line	Changes in GST
Adriamycin	100	MCF-7 human breast (Adr^r)	45-fold increase in hGSTP1
Chlorambucil	15	Walker 256 rat mammary	>10-fold increase in rGSTA3
Ethacrynic acid	2.5	MCF-7 human breast	>10-fold increase in mu-class GST
Etoposide	14	MCF-7 human breast	>10-fold increase in hGSTP1
Hepsulfam	10	MCF-7 human breast	>10-fold increase in hGSTP1
Melphalan	10	MatB13762 rat mammary(Mln^r)	10-fold increase in rGSTA3 and P1
Novantrone	5	MCF-7 human breast	>10-fold increase in hGSTP1
Oxazaphosphorine	6	MCF-7 human breast	2.7-fold increase in GST
Vincristine	11	MCF-7 human breast	>10-fold increase in hGSTP1

1.1.8.1 GSTs and Multi-Drug Resistance in Chemotherapy

Circumstantial evidence has linked the increase in specific GST isoenzymes or bulk GST activity in cells with resistance to alkylating agents, doxorubicin, and other drugs (Morrow et al., 1990; Townsend et al., 1989). However, direct evidence that GSTs are responsible for altering drug sensitivities is limited. Another catalytic activity, selenium-independent glutathione peroxidase activity, has been attributed to some isoenzymes of GST. Since some antineoplastic

agents, including doxorubicin, may exert their cytotoxic effects through the generation of free radicals and lipid peroxides, this GST-mediated reaction is a potential mechanism of resistance.

Studies using cell-free preparations of GSTs have identified a limited number of antineoplastic drug substrates of these enzymes, including chlorambucil. Whether GST levels in tumour cells are sufficient to detoxify antineoplastic drugs to a clinically significant extent is a matter of considerable debate. Gene transfer experiments using recombinant GST genes and tissue culture cells have suggested that some GST isoenzymes may confer a very modest level of resistance to melphalan, chlorambucil, cisplatin (Puchalski et al., 1990), and doxorubicin(Nakagawa et al., 1990). Other experiments have failed to confirm any consistent resistance to doxorubicin, cisplastin, or melphalan in breast cancer cells transfected with the pi class isoenzyme of GST (Moscow et al., 1989; Leyland-Jones et al., 1991). Thus, whether GSTs are involved in resistance or are makers of resistance is not clear. Additional studies are necessary to clarify the role of GSTs, if any, in drug resistance.

Studies in doxorubicin-resistant MCF-7 breast cancer cells had indicated that the level of GST was markedly overexpressed in the resistant cells and that the increase was due to an increase of one specific isoenzyme of GST (pi class GST). Furthermore, in a small subset (n=72) of patients with node-negative breast cancer who received no adjuvant hormonal therapy or chemotherapy, increased pi class GST expression was associated with significantly shorter disease-free survival as well as overall survival (Gilbert et al., 1993). Multivariate analysis indicated that the effect of pi class GST expression on prognosis was independent of tumour grade and hormone receptor status. Thus, expression or drug metabolising enzymes may be important indicators of prognosis as well as possible markers of drug and hormone sensitivity.

Resistance to Alkylating Agents

GSH forms conjugates with a variety of alkylating agents in both nonenzymatic and GST-dependent reactions (Morrow et al., 1990). Several laboratories have demonstrated an association between increased total GST levels or specific GST isozymes with resistance to drugs such as nitrosoureas (Evans et al., 1987), chlorambucil, and other nitrogen mustards(Puchalski et al., 1990; Robson et al., 1987; Buller et al., 1987; Lewis et al., 1988; Wolf et al., 1987). Additionally, increased GSH levels have been correlated with resistance to alkylating agents and cisplatin(Hamilton et al., 1990; Somfai-Relle et al., 1984). While the electrophilic cisplatin compound can react directly with GSH, it is unknown whether GSTs can catalyse this reaction. This is unresolved by conflicting results, which show a correlation between elevated expression of the pi isozyme of GST and resistance to cisplatin in some cells (Nakagawa et al., 1988; Miyazaki et al., 1990), but not others(Moscow et al., 1989).

The correlations between GSH or GST levels and drug resistance are variable. Indeed, some investigators have been unable to demonstrate a relationship between the overexpression of multiple isozymes of GST and antineoplastic resistance (Fairchild et al.,1990; Moscow et al., 1989; Leyland-Jones et al., 1991). In other studies that have compared paired parental and resistant cell lines, the magnitude of alkylating agent resistance associated with increased GST activity is often modest. While the clinical importance of GST and GSH in alkylating resistance is accordingly debated, existing preclinical data has prompted phase I trials using GST inhibitors or the GSH synthesis inhibitor BSO in conjunction with alkylating agents.

Therefore, increased intracellular GSH levels as well as increased activity of GSH-dependent enzymes have been associated with multidrug resistance in some studies, whereas others have failed to find evidence for a role of GSH or its dependent enzymes in multidrug resistance.

GST isoenzymes presence or absence might therefore account for the resistance of tumours to particular chemotherapeutic drugs. Determination of the GST isoenzyme profile of a cancer tissue could have prognostic value in the selection of treatment if the levels of expression/activity show a degree of variation comparable with that exhibited by actual patient responses. In most cases, the average level of GST was substantially elevated in the cancer tissues above the levels in normal breast tissue from the same patient. Furthermore, the relative levels of the isoenzymes were substantially more variable in the cancer samples than in the normal breast tissue, providing a plausible mechanism for the well established variable response to treatment.

1.1.8.2. GSTs in Hormone Therapy

Antioestrogens are now widely used in the management of breast cancers, either as adjuvant therapy or more recently as neoadjuvant therapy. These drugs are of particular interest and importance because of their clinical relevance, improving survival of breast cancer patients. They act mostly through the oestrogen receptor system (Katzenellenbogen et al., 1985), but the exact mechanism of their anti-tumoral effects is not completely understood. A link between the presence of oestrogen receptor in breast tumours and response to endocrine therapy has been demonstrated, but the prediction needs to be improved by additional factors. In order to understand better the mode of action and mechanism of resistance to endocrine therapy, a better knowledge of biological changes arising under tamoxifen is warranted. Although several studies exist in vitro, only a few papers deal with changes occurring in vivo, in hormonal receptor content and oestrogen-related proteins of breast carcinomas following tamoxifen administration (Allegra et al., 1980; Waseda et al.,1981; Taylor et al.,1982; Hull et al.,1983; Melchor et al.,1990; Leroy et al.,1991).

Dorion-Bonnet et al., (1993) reported that tumour patients not responding to tamoxifen therapy had wider-ranging amounts of GST-pi RNA levels (0 to 74%, median value 16%) than responsive tumour patients (0 to 5%, median value

8%).They suggested a significant relationship of tamoxifen therapy responsiveness to GST-pi RNA levels.

Soubeyran et al.,(1996) have studied a group of post-menopausal breast carcinoma patients first treated by neoadjuvant hormonal therapy (tamoxifen). Using immunohistochemistry (IHC) on pre-treatment core biopsies, they have investigated the value of ER, PR and three oestrogen-related factors [pS2, GST-pi and the oncogene c-erbB-2] as markers of hormone responsiveness. pS2, a small cysteine-rich protein of unknown function, appeared with ER to be strongly correlated with tamoxifen-induced tumour regression. This had already been suggested by others (Henry et al.,1989,1991; Schwartz et al., 1991; Hurlimann et al.,1993; Wilson et al.,1994). They were unable to show a link between response to tamoxifen and c-erb-2 or GST-pi expression contrary to previous studies (Wright et al., 1992; Nicholson et al., 1993; Dorion-Bonnet et al., 1992). They observed a significant increase in GST-pi expression and a decrease in ER and PR following tamoxifen treatment. However, they have found no relation between these variations and efficiency of hormone therapy.

1.1.9 Polymorphic Expression of GSTs

Genes coding for the glutathione-S-transferase M1 (GSTM1) and theta1 (GSTT1) proteins are polymorphic in humans and these genes are absent, or homozygous null, in 10-60% of different ethnic populations. These enzymes which are involved in the metabolism of many carcinogens, environmental pollutants, anticancer drugs are of particular interest as allelic differences may account for wide interindividual differences in sensitivity to cancer-inducing or cancer promoting compounds (Nebert, 1980).

Interindividual differences in human class mu GST are commonly observed and were first described in the early 1980s (Warholm et al., 1980; Board et al., 1981). Such variation is due to either to gene deletion, resulting in failure to express protein, or allelic variation, resulting in production of catalytically active

protein with an altered charge. The frequency of the GST mu null homozygosity is 50% in population.

Epidemiological studies suggest that individuals who are homozygous nulled at the GSTM1 locus may have an increased risk of developing various types of neoplastic disease, including cancer of the bladder, colon, lung, skin, and stomach.

Seidegard et al. (1986) provided the first evidence that an association exists between the GSTM1 null phenotype and the risk of developing lung cancer when they reported an approximate 1.6-fold increase in the frequency of nulled individuals among patients with lung cancer. It has been suggested that high GSTM1 activity is associated with a greater decrease in lung cancer risk among heavy than light cigarette smokers (Nazar-Stewart, et al., 1993). Zhong et al. (1991) suggested that GSTM1 may protect against certain types of lung cancer, such as squamous and small cell carcinoma, but not against adenocarcinoma of the lung. Similarly, Liu et al. (1991) showed that the presence of GSTM1 in human liver cytosol inhibited the ability of benzo[a]pyrene as well as aflatoxin B_1 to form adducts with calf thymus DNA.

A number of groups have found evidence that the GSTM1 null genotype is a risk factor for bladder cancer. Among cancer patients from England, Daly et al. (1993) reported that 85% of 53 patients with bladder cancer were GSTM1 null compared with 53% in healthy controls and 60% in patient controls.

Absence of GSTM1 may predispose toward stomach and colon cancer. Strange et al. (1991) suggested that nulled individuals have an approximately threefold greater risk of developing these malignancies.

Skin cancer is another malignancy where the GSTM1 null genotype may confer increased risk of disease (Heagarty et al., 1994). It has been proposed that

the protective role of the GSTM1 locus in skin cancer involves antioxidant defenses against ROS generated by UV light, rather than protection against xenobiotics.

The GST theta family is responsible for the GSH-dependent detoxification off monohalomethanes in human erythrocytes and liver (Peter et al, 1989; Hallier et al., 1993; Pemble et al., 1994). Approximately, 60-70% of the human population are able to carry out this metabolic reaction ("conjugator"), whereas the remainder are unable to perform the conjugation ("non-conjugators") (Peter et al, 1989). Further characterization of these phenotypes revealed that GSH conjugation of the industrial chemicals dichloromethane and ethylene oxide (EO), could only be catalysed by blood samples from the "conjugator" population (Thier et al., 1991; Foest et al., 1991). The monohalomethanes, EO, DCM and other man-made alkyhalides are widely used industrial methylating agents:fumigants, pesticides and solvents. Many of these substrates are known or suspected carcinogens. The null polymorphism at the GSTT1 locus may therefore modify the individual risk associated with exposure to toxic and carcinogenic chemicals.Support for this notion is provided by a study showing that in a population exposed to 1,3-butadiene, the GSTT1 null (GSTT10) individuals demonstrated a 16-fold increased frequency of sister chromatic exchange in lymphocytes than GSTT1-1 individuals (Wiencke et al., 1995).

Few data have been reported linking the GSTT1null genotype to cancer susceptibility. In a study of Australian patients with colorectal cancer, the frequency of GSTT10 homozygotes did not differ from controls. However, the nulled individuals were more common in patients diagnosed before 70 years of age than in those diagnosed after 70 years of age (Chenevix-Trench et al., 1995), suggesting that GSTT1 genotype may influence the age of onset of colon cancer. The GSTT1 null is associated with increased risk of astrocytoma and meningioma (Elexpuru-Camiruaga et al. 1995) and epithelial ovarian cancer (Sarhanis et al. 1996). However, there is no associations between GST T1 genotypes and susceptibility to lung, oral and gastric cancers (Duncan et al. 1995). The identification of a homozygous null genotype in the human theta GSTT1 gene suggests this locus is a potential candidate for cancer risk. The influence of GSTT1 null on susceptibility to a cancer will depend on causative substrates (Pemble et al. 1994). That GSTT1 null

is not a critical factor in mediating risk of cervical neoplasms implies that the detoxification/activation of mono- and dihalomethanes is not significant in the pathogenesis of cervical neoplasms (Warwick et al. 1994).

1.2 Female Breast

1.2.1 General Anatomy of Human Breast Tissue

The mammary glands are modified eccrine glands of the skin located on the anterior chest wall whose ductal and lobular units extend far into the adjacent subcutaneous fat. The gland itself is segmentally divided into 15 to 20 distinct glandular units, or lobes, each of which has a ductal orifice at the apex of the nipple.

The normal adult female breast is composed of an admixture of epithelial and stromal elements. The epithelial elements consist of a series of branching ducts which connect the structural and functional units of the breast, the lobules, to the nipple (Fig. 2) The stroma is composed of variable amounts of adipose tissue and fibrous connective tissue, and comprises the majority of the breast volume in the nonlactational state.

The nipple is transversed by multiple lactiferous ducts that range from 2 to 4 mm in diameter. These have convoluted walls (to permit high flow) and a squamous lining near the surface exit. They commonly create a pocket or vestibule, known as the lactiferous sinus, that lies at the base of the nipple. The ducts and sinuses are lined by a single or double layer of simple columnar epithelial cells. As the ducts ramify deeper into the breast parenchyma, they form smaller ductal units. These smaller ducts and ductules are lined by a two-cell layer composed of inner epithelial cells and outer, spindle-shaped mesenchymal myoepithelial cells. The basic hormonally sensitive and lactational unit of the breast is the terminal ductal-lobular unit. In the resting phase, this unit is lined by cuboidal epithelium that is surrounded by an inactive layer of myoepithelial cells.

The lobular units vary from 0.3 to 0.6 mm in diameter and are interconnected by a labyrinth of extralobular terminal ductules. These are hormonally sensitive and may be the site of intraductal hyperplasia. These ductules fuse in a cascading series to form the final 15 to 20 largest ducts of the breast that come together immediately beneath the areola (Snell, 1984; Leeson et al., 1988; Ross et al., 1988; Arey, 1974; Amenta, 1986; Hughes et al., 1988; Karcioglu and Someren, 1985; and Fechner and Mills, 1990).

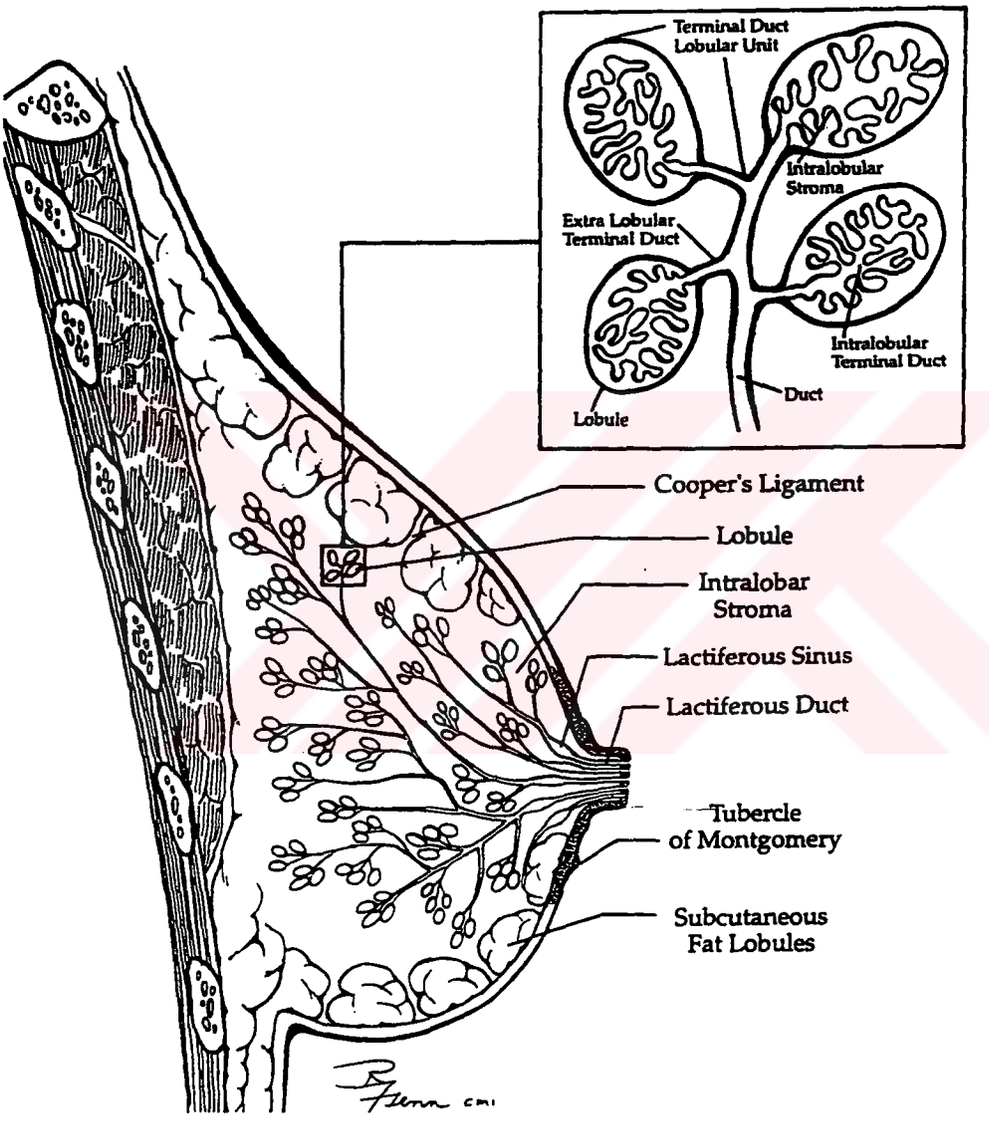


Figure 2 Anatomy of the breast (William, L. D., Spratt, J. S.; 1995)

1.2.2 Breast Tumours

Neoplasms constitute the most important, albeit not the most common, lesions of the female breast. Breast tumours may arise either from the stroma or epithelial component of the breast. Characteristic of the most common breast tumours will be summarised in this section.

1.2.2.1 Stromal tumours

The two types of stroma in the breast, intralobular and interlobular give rise to distinct types of neoplasms. Breast specific intralobular stroma is the progenitor of the breast specific biphasic tumours, fibroadenoma and phyllodes tumour. The specialised stroma may elaborate growth factors for epithelial cells resulting in the associated proliferation of the non-neoplastic epithelial component of these tumours. Interlobular stroma is the source of the same types of tumours found in connective tissue in other sites of the body (e.g. lipomas, angiosarcoma).

1.2.2.1.1 Phyllodes Tumours

Phyllodes tumours arise from intralobular stroma although they can occur at any age, most present in the six decade. Most are benign or low grade malignant tumours. Phyllodes tumours resemble fibroadenomas on histologic examination but they are distinguished from the more common fibroadenoma on the basis of cellularity, mitotic rate, nuclear pleomorphism, stromal overgrowth and infiltrative borders.

1.2.2.1.2 Fibroadenoma

Fibroadenoma is the most common benign tumour of the female breast. Multiple studies have shown that the stromal component is clonal but epithelial component is polyclonal. Fibroadenomas are more common before 30 years of age. Fibroadenomas are associated with amild increase in the risk of subsequent breast

cancer. The histologic pattern is essentially one of delicate cellular fibroblastic stroma enclosing glandular spaces lined by epithelium.

1.2.2.2 Epithelial Tumours

1.2.2.2.1 Large Duct papilloma

Most of this lesions are solitary and are found within the lactiferous ducts or sinuses. They represent papillary clonal proliferations of epithelial cells.

1.2.3 Breast Cancers

Breast carcinoma is the most common malignant tumour and leading cause of cancer death in women. The incidence is high in North America and Northern Europe and low in most Asian and African countries. In Turkey, it accounts for 28.38% of all cancers (İşcan, 1994), and is the commonest cause of death amongst women in the 35-55 age group (Bakır et al., 1993).

1.2.3.1 Risk Factors

Many risk factors have been identified, and these, together with advances in the analysis of genetic and hormonal factors, have resulted in several aetiological hypotheses. An understanding of the risk factors can help in the development of programmes directed towards the prevention of breast cancer. At present, schemes aimed at the early detection of breast cancer are being introduced.

Female sex and age

Less than 1% of all breast cancers occur in men, so being female is an important risk factor. As with all carcinomas, increasing age is another significant factor. Up to the age of 40-45 years, the rate of increase is steep; it then slows down, although the incidence of breast cancer continues to increase into old age.

Age at menarche and menopause

There is a significantly higher risk of developing breast cancer amongst women with an early age at menarche. At the other end of the reproductive life, women whose natural menopause occurs before 45 years have only half the breast cancer risk of those whose menopause occurs after 55 years. Therefore women with 40 or more years of active menstruation have twice the breast cancer risk of those with fewer than 30 years of menstrual activity.

Exogenous oestrogens

In some series there has been two to nine fold increased risk was observed for the women who had exogenous oestrogen therapy

Contraceptive agents

The various epidemiological studies have shown no or very low increase among long term users.

Age at first full-term pregnancy

Nulliparous women have an increased risk of developing breast cancer. However, among parous women protection is related to early age for the first full-term pregnancy. If the first birth is delayed to the mid or late thirties, the woman is at a greater risk of developing breast cancer than is a nulliparous woman.

Weight and diet

For women of above average weight but below 50 years of age there is little or no increased risk of developing breast cancer. However, women aged 60 or over whose weight is increased have a higher cancer risk. Diet, obviously, can be a

determinant for weight. In rodents, a high-fat diet increases the incidence of breast tumours, and international breast cancer incidence rates correlate with the consumption of fat. Although these observations suggest that a high-fat diet may be a risk factor, the evidence is not as clear as it is for weight (Willett et al., 1992). Coffee addicts will be pleased to know that there is no substantial evidence that caffeine consumption increases the risk, but studies suggest that moderate alcohol consumption is associated with a 1.5 increased risk of breast cancer.

Family history and genetic factors

Breast cancer is common, thus a history of a relative having breast cancer can be found in at least 10% of new cases. However, a proportion of these will be sporadic cancers and not due to familial (inherited genetic) factors. The risk of developing breast cancer is increased in first-degree relatives (e.g. sister, daughter) of breast cancer cases, particularly if that person is pre-menopausal. For example, the risk increases to nine-fold for first-degree relatives of premenopausal women with bilateral breast cancer. Up to five-fold increases in risk have been found for women with multiple first-degree relatives with breast cancer.

Geographic variation

There is a marked variation in breast cancer rates between different countries. The highest rates are in North America, North-west Europe, Australia and New Zealand, with the lowest rates in South-east Asia and Africa. Several factors probably contribute to this difference: age at menarche, age at first full-term pregnancy, age at menopause and post-menopausal weight. The length of time between age at menarche and first pregnancy may be quite short in some of these low-incidence countries.

Atypical hyperplasia

Women with benign breast disease whose breast biopsies show atypical epithelial hyperplasia have a definite increased risk of developing breast cancer. Ordinary epithelial hyperplasia is associated with a slightly increased risk. The risk is augmented by a family history of breast cancer.

1.2.3.2 Diagnosis

Clinical examination particularly palpation is the time-honoured method for the detection and evaluation of breast disease however, its sensitivity and discriminatory power are limited. Only 60% of the tumours detected by mammography are palpable. The wide spread use of mammography has radically changed the diagnostic approach to breast cancer, extremely small tumours (1 to 2 mm) can be detected with this technique that relies primarily on the presence of calcification. The incidence of calcification in breast carcinoma is about 50-60% and this incidence is 20% in benign breast disease. However, a negative mammogram does not rule out the possibility of the presence of carcinoma. About 20% of palpable tumours are not detectable with mammography.

In several developed countries with a high incidence of breast cancer, such as the UK, screening programs for the detection of early breast cancer have been or are being introduced. Trials in Sweden and the USA strongly suggest that women whose cancers have been detected by regular mammographic screening have an increased survival rate which means that patients will live longer from their time of diagnosis. This is because the tumours are detected when they are either pre-invasive (in-situ carcinoma) or invasive but small, with less risk of metastasis and the treatment is initiated at the time of early detection. Unscreened women present when the tumour has grown to a size sufficient to be felt, at which stage there is a higher probability of metastases. The apparent benefits of screening mammography are a true reduction in mortality by early treatment.

The American Cancer Society recommendation includes annual physical examination beginning at age 40 and screening mammography at one- to two-year intervals between the ages of 40 and 50 and every year at age 50 and older. In the UK, women between the ages of 50 and 64 are invited to attend for breast screening by mammography every 3 years. Suspicious features on the X-ray image, such as microcalcifications, which are calcium particles of various size and shape measuring 100 microns to 1 mm in diameter and numbering greater than 4-5 per cubic cm, and areas of architectural distortion of dense tissue, are further investigated by ultrasound and clinical examination, with cytology of aspirated cells and histology of biopsy samples providing the definitive diagnosis.

More than 90% of all breast cancers that are detected by annual screening with mammography and physical examination are identified by mammography. Microcalcifications are seen in approximately 60% of cancers detected by mammography. Approximately 5% of noncalcified cancers present as areas of architectural distortion of dense tissue.

1.2.3.3 Classification of Breast Carcinoma

The two key determinations to make in the morphologic study of breast carcinoma are

(1) Whether the tumour is confined to the glandular component of the organ (in situ) or whether it invades the stroma (invasive carcinoma) and
(2) Whether it is of ductal or lobular type.

1.2.3.3.1 Non-invasive Carcinomas

Virtually all breast carcinomas are adenocarcinomas derived from the epithelial cells of the ducts or glands.

The term 'non-invasive' means that the malignant cells are confined to either the ducts or the acini of the lobules, with no evidence of penetration of the

tumour cells through the basement membranes around these two types of structures into the surrounding fibrous tissue. There are two forms of non-invasive carcinoma:
- ductal carcinoma in situ
- lobular carcinoma in situ.

1.2.3.3.1.1 Ductal Carcinoma in situ

Ductal carcinoma in situ can occur in both pre- and post-menopausal women, usually in the 40-60-year age group. It can present as a palpable mass, especially if extensive and associated with fibrosis. If the larger ducts are involved, presentation can be as a nipple discharge. Pure ductal carcinoma in situ accounts for about 5% of breast carcinomas which present clinically (Rosner et al., 1980).

The size of the area involved in the breast can range from 10-80 mm in length. It is usually unifocal, being confined within one quadrant of the breast, although multicentricity can occur with larger lesions.

Most cases of ductal carcinoma in situ have been treated by mastectomy, so it is difficult to know the fate of these lesions if left. Estimates of residual carcinoma changing from non-invasive to invasive range from one-third to one-half, based on studies where only local excision has been performed.

1.2.3.3.1.2 Lobular Carcinoma in situ

Lobular carcinoma in situ (intralobular carcinoma) occurs predominantly in pre-menopausal women. If it is found after the menopause it is usually associated with an infiltrating tumour. Lobular carcinoma in situ accounts for about 6% of all breast carcinomas. A major problem is that it does not present as a palpable lump and is usually found in biopsies removed because of cysts or other palpable benign lesions. A further important clinical feature is that it is often multifocal within the one breast and is frequently bilateral (Lattes, 1980; and Rosner et al., 1980).

About one-quarter to one-third of all patients with lobular carcinoma in situ who are treated by biopsy alone will go on to develop an invasive carcinoma. This may occur in either or both breasts and there may be a long time interval.

1.2.3.3.2 Invasive Carcinomas

Carcinoma of the breast originates from the epithelium of mammary ducts and acini; however, the studies by Wellings and colleagues (1974) have shown that a large percentage of breast carcinomas, both ductal and lobular, take origin in the terminal duct lobular unit rather than from larger ducts.

An 'invasive' tumour is one whose cells have broken through the basement membrane around the breast structure in which they have arisen, and spread into the surrounding tissue. Invasive carcinomas are categorised into different histological types.

The histological types of invasive carcinoma and their relative incidence for palpable tumours are:

- invasive ductal no special type (NST) (70-80%)
- invasive lobular (5-10%)
- mucinous (1-6%)
- tubular (2%)
- medullary (1-5%)
- papillary (<1%)
- others (<1%)

There is a higher frequency of tubular carcinoma in mammographically detected tumours.

Carcinomas vary in size from less than 10mm in diameter to over 80mm, but are often 20-30mm at presentation. Clinically, they are firm on palpation and

may show evidence of tethering to the overlying skin or underlying muscle. The skin also shows *peau d'orange*, dimpling due to lymphatic permeation. The nipple may be retracted due to tethering and contraction of the intramammary ligaments. The macroscopic appearance of the tumours tends to depend on the amount or type of stroma within the carcinoma. The term scirrhous implies that there is a prominent fibrous tissue reaction, usually in the central part of the tumour. This results in the carcinoma having a dense white appearance, which grates when cut. Yellow streaks may be seen; these are due to the presence of elastic tissue within tumour. Carcinomas with a prominent stromal reaction usually have irregular edges, extending into the adjacent fat or breast parenchyma.

1.2.3.3.2.1 Invasive Ductal Carcinoma (NST)

Invasive duct or ductal carcinomas comprise the majority (up to 85%) of infiltrating breast carcinomas (Fisher et al., 1975; Azzopardi et al., 1982; Page and Anderson, 1987; and Rosen and Oberman, 1992). The size of the tumours varies between patients (average 1 to 2cm in diameter). They can occur in both pre- and post-menopausal women.

On palpation, they may have an infiltrative attachment to the surrounding structures with fixation to the underlying chest wall, dimpling of the skin, and retraction of the nipple. Histologically, the tumour cells are arranged in groups, cords and gland-like structures.

1.2.3.3.2.2 Invasive Lobular Carcinoma

This is a distinct morphologic form of mammary cancer that probably arises from the terminal ductules of the breast lobule. Although making up only 5 to 10% of breast carcinomas (Donegan and Perez-Mesa, 1972; Martinez and Azzopardi, 1979; and Dixon et al., 1983), invasive lobular carcinomas are of particular interest for at least two reasons: (1) They tend to be bilateral far more frequently than those arising in ducts and (2) while invasive ductal carcinomas

usually form at one focus in the breast, they tend to be multifocal within the same breast. Invasive lobular carcinomas have abundant fibrous stroma, so that macroscopically they are always scirrhous.They occur in post-menopausal women.

Histologically the cells are small and uniform and are dispersed singly, or in columns one cell wide in dense stroma. The cells infiltrate around pre-existing breast ducts and acini, rather than destroying them as occurs with invasive duct carcinomas.

Tumours not detected by mammography often belong to this category because calcifications frequently are not present and the pattern of invasion is diffuse, without margination (LeGal et al., 1992).

The prognosis, according to various studies, is not different from that for infiltrating ductal carcinoma of no specific type when tumours of similar stage are compared.

1.2.3.3.2.3 Mucinous Carcinoma

Mucinous carcinomas (also known as colloid, mucoid and gelatinous carcinomas) usually arise in post-menopausal women. Its growth is slower than that of the usual invasive ductal carcinoma.

Macroscopically, the tumours are well circumscribed and have a soft, grey, gelatinous cut surface. They vary in size from 10-50mm in diameter. Since there is no dense stroma and the edges are rounded, these tumours do not cause retraction of the nipple or tethering of the skin.

These carcinomas comprise small nests and cords of tumour cells, which show little pleomorphism, embedded in large amounts of mucin. The latter is composed of neutral or weakly acidic glycoproteins, which are secreted by the tumour cells and are different from the proteoglycans of the stroma.

The survival of women with mucinous carcinomas is better than that of those having invasive duct or lobular carcinomas.

1.2.3.3.2.4 Tubular Carcinoma

The distinctive characteristic of tubular carcinoma is well-delineated tubules or glands separated by a fibrous stroma. Because of its bland appearance it has been termed well-differentiated carcinoma. They are usually small lesions, less than 10mm in diameter, and are firm, gritty tumours with irregular outlines. Tubular carcinomas form 1-2% of invasive carcinomas but constitute a higher proportion of screen-detected tumours. Histologically, they are composed of well-formed tubular structures, the cells of which show little pleomorphism or mitotic activity. The stroma is dense, often with elastosis. Patients with tubular carcinomas do extremely well-better than those with well-differentiated invasive duct carcinomas.

1.2.3.3.2.5 Medullary Carcinoma

Medullary carcinoma represents about 5% all invasive carcinomas. The distinguishing gross characteristics consist of a globoid, homogeneous, soft, pinkish gray mass, with well-defined margins. When haemorrhage or necrosis has occurred, dark red or yellow areas may be observed.

Medullary carcinomas are circumscribed and often large with areas of necrosis. Histologically, they are composed of large tracts of confluent cells with little stroma in between them. The cells show quite marked nuclear pleomorphism, and mitotic figures are frequent. Despite the aggressive cytological features of these tumours, the patients have a significantly better 10-year survival than women with invasive duct carcinomas.

1.2.3.3.2.6 Papillary Carcinoma

Invasive papillary carcinoma represents between 0.3% and 3% of all breast carcinomas (Fisher et al., 1980; Azzopardi, 1983). This tumour is more frequently found in post-menopausal women. They are usually circumscribed and can be focally necrotic, with little stromal reaction. The tumours are in the form of papillary structures, and areas of intraductal papillary growths are usually found. The prognosis of these carcinomas is probably better than that of the much more common invasive duct carcinoma (Fisher et al., 1993).

1.2.3.4 Histological Differentiation

Histologic and biologic features of breast cancer also influence prognosis. One of the most important is histopathologic grade. The two widely used system to determine the histological differentiation of breast carcinoma have been those of Bloom and Richardson and Black, the first based mainly on architectural features and the second on the degree of nuclear atypia.

1.2.3.5 Hormone Receptors

Determination of oestrogen and progesterone receptors on tumour tissue is presently part of the standard of practice for selection of treatment and a prognostic guide for patients with invasive ductal carcinoma of the breast. Not much correlation exist between the type of breast carcinoma and presence of hormone receptors. However, most series have shown that most medullary carcinomas and in situ duct carcinomas of the comedo carcinoma type are negative, whereas mucinous carcinomas have the highest rates of possibility. Generally, oestrogen receptor concentrations are lowered in tumours of premenopausal women than those of postmenopausal women. The presence of receptor proteins indicates retention of the regulatory controls of the mammary epithelium. Tumours that are better differentiated are predominantly ER-positive and PR-positive and are associated with better prognosis (Donegan, 1992). Some studies have shown that about 60% of women with ER-positive tumours respond to hormone manipulation, in contrast with 5% to 10% of women with ER-negative tumours. Progesterone

receptors are equally valuable; the presence or absence of PR may even be a better predictor for endocrine therapy. Still, there is controversy concerning its prognostic value as an independent variable. It requires evaluation in combination with age and tumour differentiation. In Stage I disease, ER and tumour size are useful for identifying a group at high risk for early recurrence and poor chances of survival. For Stage II the number of involved nodes and the PR are more important (McGuire, 1986).

The presence of oestrogen receptors within a carcinoma indicates that the tumour cells have a higher degree of functional differentiation. It is thus not surprising that women whose tumours are oestrogen-receptor-positive have better 5- and 10-year survival figures than those whose carcinomas are oestrogen-receptor-negative and they are more likely to benefit from tamoxifen, an oestrogen receptor antagonist.

1.2.3.6 Stage

Staging refers to classification of breast cancer by anatomic extent. The premise that underlines staging is that breast cancer progress anatomically in an orderly manner and that its progression is related to prognosis.

For breast cancer classification, the TNM system, which has been accepted by The American Joint Committee on Cancer in 1992, is shown in Table 4. The TNM system is based on description of primary tumour (T), the regional lymph nodes (N), and distant metastases (M). The subcategorises of T, N, and M were then grouped in various combinations to describe four stages (Table 7). The measurement of size applies only to invasive cancer. Hence, only the invasive component of a tumour is considered in measuring its diameter; any in situ component is ignored.

Table 7. TNM Staging System for Breast Cancer ,1992 (The Cancer of the Breast, 1995)

TNM Stage	Primary tumor (T) cm	Nodal metastases (N)*	Distant metastases (M)
0	In situ	No	No
I	≤2	No	No
IIA	≤2	Yes	No
	>2≤5	No	No
IIB	>2≤5	Yes	No
	>5	No	No
IIIA	≤5	Fixed	No
	>5	Fixed or mobile	No
IIIB	Skin or chest wall	Any	No
	Any	Internal node	No
IV	Any	Internal node	Yes^^

*Pertains to movable ipsilateral axillary nodes unless otherwise stated
^^ or supraclavicular nodes

Non-invasive (in situ) tumours are specified as such and without further reference to stage. Stage I means an invasive cancer confined to the breast without involvement of the skin or chest wall. Stage II indicates such a tumour with early spread to axillary nodes, and Stage IV indicates the presence of distant metastases. Stage III includes variations of local and regional advancement that are between Stage II and IV.

The overall 5-year survival rate for stage I cancer is 80%; for stage II, 65%; for stage III, 40%; and for stage IV, 10%. It should be noted that recurrence may appear late, even after 10 years, but with each passing year free from disease, the prognosis improves. In the overall view of breast cancers, the 10-year survival is still no more than 50%.

1.2.3.7 Treatment of Breast Carcinoma

The therapy of breast carcinoma includes surgery, radiation therapy, hormone therapy and chemotherapy depending on the type and extend of the disease. Surgical therapy comprises a wide variety of options which include radical mastectomy, partial mastectomy, total mastectomy and modified radical mastectomy. Radiation therapy has been employed as a postoperative adjunct, sometimes as the primary treatment and for the control of locally recurrent disease. Although local control of breast carcinoma is important, chemotherapy of metastatic breast cancer are also critical for patients. Of the common solid tumours, breast cancer is one of the most responsive to systemic therapy. Two types of systemic modalities are effective in the treatment of breast cancer: chemotherapy, which consists of agents that are specifically cytotoxic; and hormone therapy, which involves using agents or modalities that alter the endocrine milieu and therefore affect tumour cell growth. Chemotherapy and endocrine therapy effectively prevent relapse and prolong the survival of patients with newly diagnosed primary cancer who have no grossly detectable recurrence. This is called adjuvant systemic therapy. In patients who have grossly detectable metastatic disease, the agents are used to improve quality of life by reducing symptoms as a result of shrinkage of the tumour masses.

Disseminated breast cancer currently is not curable, and patients at this stage of the disease will die from progressive tumour. Nevertheless, metastatic breast cancer is very treatable. Patients whose disease responds, survive longer and enjoy better quality of life. With continued treatment, the disease becomes resistant, tumour progresses, and therapy must be changed. Continuing changes lead to increased toxicity of treatment, and decreases in rate and duration of response.

Not all chemotherapeutic agents are active against every type of tumour. The most active, and therefore most commonly used, chemotherapeutic drugs for breast cancer include the alkylating agents (cyclophosphamide, L-phenylalanine

mustard, thiotepa), the antitumor antibiotics (doxorubicin), the antimetabolites (methotrexate, 5-fluorouracil), and certain vinca alkaloids (vinblastine). With such a variety of drugs that are active as single agents against advanced breast cancer, combinations of drugs can be created that have independent mechanisms of action and varying toxicity.

Normal breast epithelial cells and their malignant counterparts are also very sensitive to the effects of steroid hormones, specifically oestrogen's, progesterone's, and androgens. Oestrogen primarily appears to stimulate normal ductal growth, whereas progesterone is responsible for lobulo-alveolar development.

Knowledge of the ER content of a breast cancer is important for two reasons. Tumours with high ER or PR content are better differentiated, and patients with these tumours have a better prognosis. In addition, tumour concentration of ER and PR protein is strongly predictive of response to endocrine therapy.

ER and PR positivity diminish with progression of the malignancy (Kamby et al., 1989). Differences in the receptor content between the primary lesion and metastatic sites have been observed. When changes in receptivity occur, they are almost always in the direction from positivity to negativity, implying tumour dedifferentiation, losing endocrine responsiveness (Tinnemans et al., 1990). Some observed changes in receptor status may be induced by treatment. It appears that hormone therapy has a greater tendency to induce changes from ER positive to ER negative status than does cytotoxic chemotherapy (Nomura et al., 1985; and Hawkins et al., 1990), which implies that chemotherapy kills cells indiscriminately, regardless of their receptor status, leaving the same proportion of ER-positive and ER-negative cells (Kardinal et al., 1986). However, hormone therapy selectively interferes with ER-positive cells. Ingle and colleagues (1986) reported that tamoxifen is the primary hormonal treatment of choice for premenopausal women as well as for postmenopausal women with hormonally responsive metastatic disease.

1.3 Immunohistochemical staining

Immunohistochemistry is the application of immunologic principles and techniques to the study of cells and tissues. The original method, devised by Coons, consisted of labeling with a fluorescent probe an antibody raised in rabbits and searching for it (and therefore for the antigen against which the antibody was directed) in tissue sections examined under a fluorescent microscope following incubation. The technical improvements that supervened in subsequent years have been responsible for these methods, becoming a staple of the histopathology laboratory.

Several procedures available, the two most commonly used at present being the peroxidase-anti peroxidase immune complex method and avidin-biotin immunoenzymatic technique. In this study, avidin biotin technique was used. The steps of this technique are shown in figure 3.

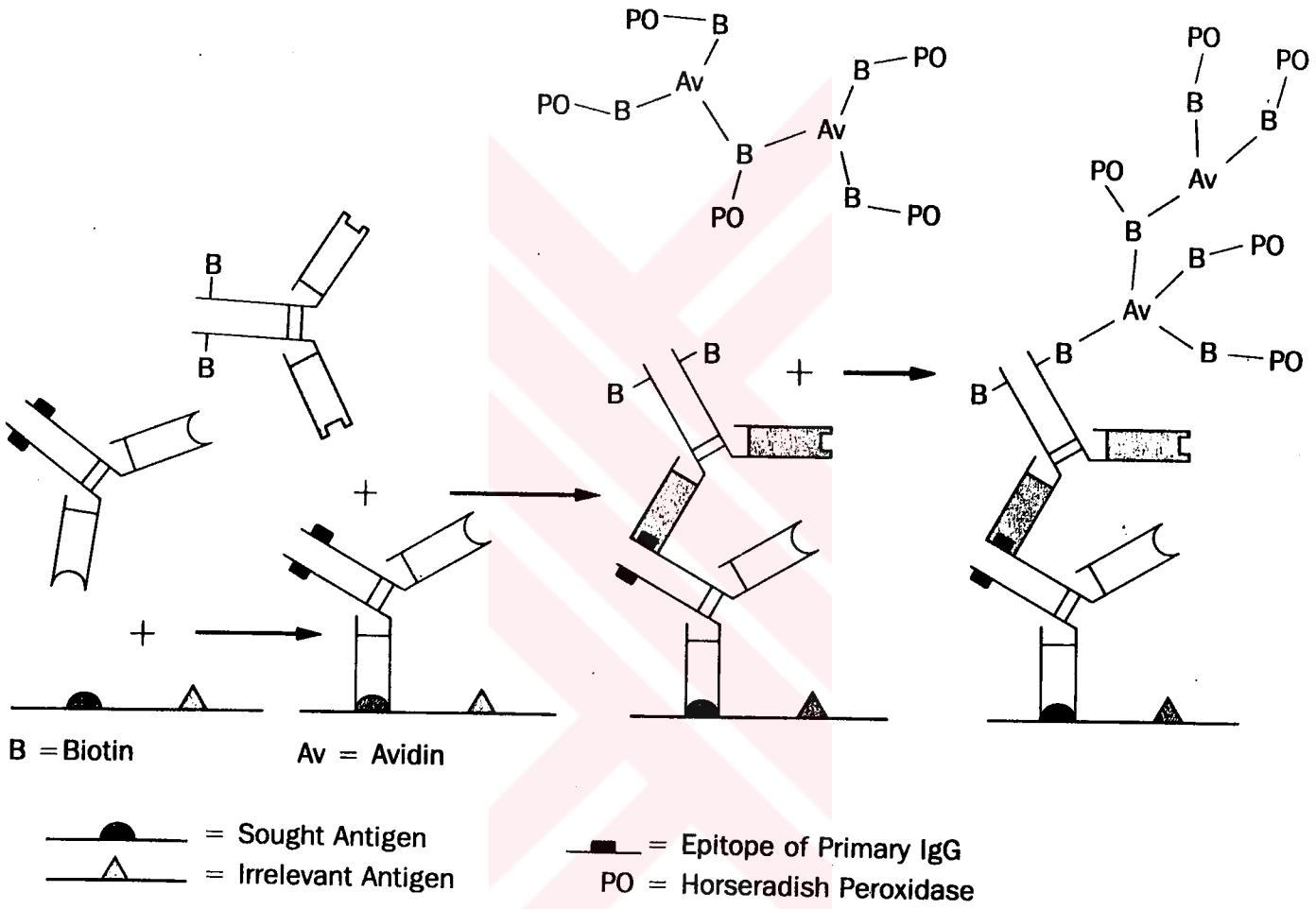


Figure 3 Avidin-biotin immunoenzymatic technique (Lawrence D. True, 1994).

Avidin is a basic glycoprotein of molecular weight 68 kiloDaltons (kD) which has an affinity for the small (244D), water-soluble vitamin biotin (vitamin H). Various methods have been devised to exploit the high efficiency and specificity of the avidin -biotin reaction. It is possible to conjugate up to 150 biotin molecules to one antibody molecule, so that the biotinylated antibody can bind to more than one avidin molecule (Robinson, Ellis and Maclennan, 1990) thereby enhancing the sensitivity of the reaction. However, avidin has two distinct disadvantages for use in immunocytochemistry. It has a high isoelectric point of approximately 10 and therefore is positively charged at neutral pH, so it may bind nonspecificaly to negatively charged structures such as the nucleus. The other problem is that as avidin is a glycoprotein it reacts with molecules such as lectins via the carbohydrate moiety, both of these difficulties lead to a reduced specificity of the reaction. These two problems can be solved with the substitution of streptavidin for avidin. Streptavidin is a protein of molecular weight 60kD isolated from the bacterium *Streptomyces avidinii* and, like avidin, has four high affinity binding sites for biotin. Also, streptavidin has an isoelectric point close to neutral pH, and as it is not a glycoprotein it does not bind lectins (Beesley, 1993).

Various labels are available for the visualisation of the antigen-antibody reaction and these include fluorescent labels, colloidal metals and enzymes. Immunofluorescent techniques were the original labelling methods used in immunocytochemistry, with fluorescein being currently the most widely used fluorochrome. However, for retrospective studies the disadvantages definitely outweigh any advantages of this technique, a major problem being fixation-induced background staining, which is why this technique is usually only used on fresh-frozen tissue. Other problems include the non-permanence of the fluorescein label due to its instability and the inability to counterstain. Colloidal gold is the most popular metal label in use and is enhanced using silver in the immunogold-silver staining procedure, which produces a black end-reaction product. This method suffers from the formation of fine silver deposits in the background, especially in inexperienced hands, and can be confusing when one is trying to identify the antigen. Colloidal gold has much wider usage with electron microscope.

Enzymes are the most widely used labels in immunocytochemistry because they produce a stable coloured reaction product suitable for light microscopy. Horseradish peroxidase (HRP) is the most commonly used immunocytochemical enzyme label in combination with the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) which produces an insoluble, dark brown reaction end-product. However, the removal of endogenous peroxidase can be problematic and requires the use of a methanol-hydrogen peroxide mixture for removal. Alkaline phosphatase is the most widely used alternative to HRP and can be used with a variety of chromogens, fast red, hexazotised new fuchsin, fast blue and tetrazolium, usually giving a red or blue colour. This has gained popularity in areas where blocking endogenous peroxidase or the presence of brown pigment makes interpretation difficult. Also, the removal of endogenous enzyme is easily achieved by adding levamisole to the developing solution.

The steps of immunohistochemical staining with avidin is peroxidase complex can be summarised as follows:

i) Prevention of non-specific background staining: Free immunoglobulins within the tissue may cause non-specific background staining and interfere with the immunohistochemical reaction. Non immune animal serum which effectively binds to free immunoglobulin within the tissue is used to overcome this problem.

ii) Application of the primary antibody: A primary antibody specific for the antigen under investigation is applied to the tissue sections.

iii) Application of the secondary antibody: A biotinylated secondary antibody that is specific for the primary antibody is applied to the tissue sections.

iv) Application of the avidin peroxidase complex: Avidin-biotin complex forms in this step.

v) Application of the substrate: A substrate for the enzyme that will produce a coloured end product is applied to the tissue sections in this step.

vi) Counterstaining: A nuclear counterstain (Haematoxylin) is applied so that the localisation of antigen within the tissue can be determined.

It is very important that extensive washes in buffer are performed between each stage to ensure that the previous reactant is totally removed before the next stage begins. Endogenous enzyme activity must also be blocked at some stage if an enzyme label is being used. For peroxidase labels the sections are treated with a hydrogen peroxide solution at the beginning of the procedure, whereas for alkaline phosphatase systems the levamisole block is added to the substrate solution used to visualise the label towards the end of the technique.

However, the immunohistochemical detection of an antigen can be influenced by many variables, including the absolute level of antigen, affinity of the antibody for the antigen, duration of the incubation, sensitivity of the detection system and the consequences of fixation. Also, the signal given in an immunohistological assay is not linear with the antibody concentration (or with any other variable), and with a given set of conditions there will be a specific threshold below which no signal is obtained (Hall and Lane, 1994).

The major artefact induced by fixation, as far as immunohistochemistry is concerned, is the masking of tissue antigens. Time, temperature, nature of the fixative used, fixative concentration and the availability of nearby proteins to be cross-linked are the variables related to the extent of masking. Proteolytic enzymes such as trypsin, pepsin and pronase are well known agents that can restore the accessibility of antibodies to epitopes previously masked by fixation (Huang, Minassian and More, 1976). However, enzymes have a limited range of effectiveness in paraffin sections and have been reported to possibly only break surface loops (Cattoretti, Pileri, Parravicini, Becker, Poggi, Bifulco, Key, D'Amato, Sabatini, Feudale, Reynolds, Gerdes and Rilke, 1993). As a result heat-mediated antigen retrieval is now often the method of choice, either using microwave ovens or autoclaving ("pressure cooking").

Autoclave pretreatment ("pressure cooking") which is used in this study, involves the immersion of dewaxed slides into superheated citrate buffer. Whilst the microwave oven method allows good reproducible results for many antigens, the

limited numbers and the constant attention required to ensure that the sections do not dry make this method very time-consuming. The alternative, large batch microwaving does suffer from inconsistencies. Miller et al, in 1995 noted that occasionally the optimised microwave oven heating time was unable to consistently recover certain antigens. The pressure cooking method does not suffer from such inconsistencies and furthermore, is far less time-consuming. It has been proposed that the presence of citrate ions at the temperature of superheated steam (120°C to 130°C) is necessary for the antigen unmasking (Bankfalvi et al, 1994). Another author reports that further protein denaturation together with the rupture of some of the aldehyde cross-links is the mechanism of action (Norton et al, 1994). However, an important point to bear in mind concerning antigen retrieval is that because it markedly alters antigen detection thresholds, it may lead to situations where many cells become falsely positive (McKee, Hobbs, Hall, 1993), so affecting the results obtained.

In order for the immunocytochemical technique to be as sensitive as possible, all the assay parameters need to be optimized. This means that for each monoclonal and/or polyclonal antibody being used, the optimum antibody concentration, optimum antibody incubation time and incubation temperature must all be determined. Also, the most appropriate form of antigen retrieval should be determined along with exposure times, concentrations and buffers to be used. Steps should also be taken to ensure that reagents do not evaporate off the sections during incubation (this is likely with the small quantities being used) such as by saturating the incubation atmosphere with tris buffered saline (TBS), and that the washing steps are extensive enough to remove all unbound reagent before the next step.

A problem at any stage in the immunocytochemical procedure (especially the early stages) can lead to false negative or positive results or high background staining levels, making analysis impossible. In order to ensure that the technique is running correctly each time it is performed, control sections should always be included. These should consist of two histological sections of tissue that is known to be positive for the target antigen, one of which is taken through the whole technique

normally (positive control) and one of which has no primary antibody applied (negative control) but is otherwise treated as the positive control and the test sections. The use of these controls each time, ensures that the results obtained are valid and reproducible, and goes some way towards providing an element of inter-assay quality control.

1.4 Scope of This Work

The GST isoenzymes composition has been detected in a variety of human normal and tumor tissues. Normal human tissues exhibit considerable variation in isoenzyme content. Human tumours from a variety of tissues, including colon, breast, lung, and stomach have previously been shown to express high concentrations of GST pi in a majority of the samples, with the other isoenzyme families which are alpha, mu and theta having received less attention. The class theta GST isoenzymes have proven to be more difficult to purify than the other classes of GSTs because they are labile and are the least abundant family.

In spite of their toxicological significance and important role in carcinogenesis, relatively little is known about the class theta enzymes where very few investigations on the localization of the class theta GSTs have been performed owing to low amounts and instability of the purified isoenzymes. To date, GST theta expression has not been detected by immunohistochemical technique in normal and tumoural human breast tissues. Immunohistochemical staining allows ready assessment of the distribution of GSTs within tissues at a cellular or subcellular level.

Differences in GST isoenzyme concentrations in tumour cells could result in differential response to anti-cancer drugs. International differences in GST isoenzyme expression in normal and tumour tissues may, therefore, be an important factor in interindividual differences in susceptibility to carcinogens and response to cancer chemotherapy. Increased expression of specific GST isoenzymes in tumour cell, particularly those that have become resistant to anti-cancer drugs, has suggested a role for these proteins in the development of resistance to

chemotherapy. As breast cancer is a major cause of morbidity and mortality in women and considering the established role of GST isoenzymes in carcinogenesis and drug detoxification as well as the reported polymorphic expression of hGSTT1-1 and hGSTM1-1, it is of special interest to identify and study GST alpha, mu, pi, theta in normal and tumoural human breast tissues.

Accordingly, normal and tumoural breast tissues were compared in terms of their GSTs isoenzyme distributions and expression levels considering the prognostic factors which were patient's age, tumour stage, parity, menopause, ER, smoking, microcalcification status by immunohistochemical technique.

The expression levels of GST isoenzymes in human normal breast tissues and carcinomas were correlated with the prognostic factors by statistical method. The effect of chemotherapy and hormonal therapy on the GSTs isoenzymes expression levels was determined by statistical analysis.

Subsequently, the GSTs from the crude extracts of matched breast normal and tumour tissues were compared in terms of their immunological properties using western blotting with monoclonal and polyclonal antibodies.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Acrylamide, hydroxymethyl aminomethane (Tris base), sodium dodecylsulfate (SDS), SDS-PAGE molecular weight markers, bromophenol blue, glycine, glycerol, Tween-20, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), mercaptoethanol, bromophenol blue, ammonium persulfate (APS), coomassie brilliant blue R-250, N,N'-methylene-bisacrylamide (Bis), N, N, N', N'-tetramethylenediamine(TEMED), bovine serum albumin (BSA), paraformaldehyde, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF), Sodium bicarbonate, copper sulfate, sodium potassium tartarate, sodium hydroxide, sodium carbonate, 3, 3'-diaminobenzidine (DAB), hydrogen peroxide, sodium citrate, citric acid, sodium chloride, dibutylthalate in xylene (DPX) were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

Monoclonal antibody against hGSTT1-1 (GSTE1-1A2.2) were the kind gift of Dr. E. Juronen, Tartu, Estonia. Polyclonal antibodies against hGST alpha, mu, pi raised in rabbit were purchased from Biotrin International Limited, Dublin, Ireland.

Swine anti-rabbit immunoglobulin biotinylated, Goat anti-mouse immunoglobulin biotinylated, Normal Swine Serum (NSS), Normal Goat Serum

(NGS), Streptavidin Biotin Complex/Horseradish Peroxidase (StreptABC/HRP) were purchased from Dako, Denmark.

Amplified alkaline phosphatase goat anti-rabbit immun-blot assay kit, sequi-blot polyvinylidene difluoride membrane (PVDF) were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.

3-Aminopropyltriethoxysilane (APES) was purchased from BDH, England. Glacial acetic acid, ultra pure methanol, Ciocalteu's Folin phenol reagent, xylene, ethyl alcohol hydrogen chloride were from E.Merck, Darmstadt, Germany.

Hematoxylin and Eosin solutions, Paraffin (M.P. 56-57°C) were purchased from Surgipath, England.

2.2 Methods

2. 2. 1 Tissues

Tissues of two different patient populations were used in this study. For western blotting analysis, 21 samples of cancerous breast tissue at lump resection, along with a portion of surrounding normal tissue, removed as a precaution against local metastases, as well as matched far neighbor of tumor tissue samples where obtained from The Demeteveler Oncology Hospital – ANKARA, and stored at -80°C. A portion of each tissue was examined by a pathologist at the source facility and confirmed as being cancerous or normal. Cancer samples selected for this study were invasive ductal carcinomas belonging to patients of two groups; subjected and not subjected to chemotherapy treatment. For immunohistochemical studies, tissues from 50 patients who were operated at Liverpool Royal Hospital Trust were used. Operation material was examined by a pathology technician in each case and three tissue samples were taken by gross examination. (1) from tumour tissue, (2) from the

edge of the tumour and normal breast parenchyma, (3) from normal parenchymal breast tissue.

Clinical parameters obtained from patients were as follows:

1-Parity

2-Age (<50 and >50)

3-Menopausal status (pre- and post-menopausal)

4-Tumour size (T1 (<2.0 cm), T2 (2.1-5 cm), T3 (>5 cm) and
T4 (Tumour of any size with direct extension to chest wall or skin)

5-Tumour stage

6-Status of the axillary lymph nodes
negative(no metastases in nodes)
positive (metastases in nodes)

7-Chemotherapy status (untreated patients and treated patients)

8-Hormone therapy status (untreated and treated patients)

9- Estrogen receptor status
(positive (ER +), negative (ER -))

10-Smoking (non-smoker, smoker)

11-Mammographic findings
Microcalcification +, Microcalcification -,

The immunohistochemical study was performed blinded to the aformentioned parameters.

2.2.2 Preparation of Cytosols from Matched Normal and Cancer Human Breast Tissues

Tissues weighing about 1.0 – 5.0 g were slightly thawed and all the subsequent steps were carried out at 4°C. The thawed tissues were minced with scissors and homogenized in 2 vol. (v/w) of TED buffer (10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 1 mM DTT, and 100 μM PMSF) using a Potter-Elvehjem homogenizer coupled with a motor (Black and Decker, V850, multi-speed drill)-driven Teflon pestle at 2400 rpm (5 to 10 passes). The homogenate was centrifuged at 12,000g for 25 minutes. The supernatant fraction was passed through two layers of cheesecloth and the pellets were discarded. The filtrates were centrifuged at 134,000g for 50 minutes and the supernatants obtained were passed through cheesecloth to remove floating lipid materials. The filtrate thus obtained was referred to as cytosolic fraction. Small aliquots of 0.5ml were stored at -80°C to be used later in protein determination, SDS-PAGE, and Western blotting.

2.2.3 Protein Determinations

The protein concentration of cytosolic fractions was determined by the method of Lowry *et al.* (1951) using crystalline BSA as a standard. Aliquots of 0.1 to 0.5 ml of 1:20 diluted breast cytosol were taken into test tubes and were completed to a final volume of 0.5 ml with distilled water. Then, alkaline copper reagent was prepared by mixing 2% copper sulfate, 2% sodium potassium tartarate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 2.5 ml of the alkaline copper reagent was added to each tube, mixed by vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.25 ml of 1 N Folin Phenol reagent was added to each test tube, mixed immediately within 8 seconds by vortex and incubated 10 minutes at 50°C in water bath. The intensity of color developed in each tube was measured at 660 nm.

The protein concentrations in the crude extracts were calculated from a standard calibration curve that was constructed from the corresponding OD_{660nm}

values of BSA standards (0 to 200 μg). The protein concentrations in the prepared breast cytosols were found to be in the range of 2.0 to 10.0 mg/ml.

2.2.4 SDS-Polyacylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis, in the presence of the anionic detergent SDS, was performed on 4 % stacking gel and 12 % or 15 % separating gel in a discontinuous buffer system as described by Laemmli (1970). The seven proteins given below were used as molecular weight standards.

- Bovine Albumin (M_r 66000)
- Egg Albumin (M_r 45000)
- Glyceraldehyde-3-Phosphate Dehydrogenase (M_r 36000)
- Carbonic Anhydrase (M_r 29000)
- Trypsinogen (M_r 24000)
- Trypsin Inhibitor (M_r 20100)
- α-Lactalbumin (M_r 14200)

2.2.4.1 Preparation of Reagents

(A) Stock Separating Gel Buffer (1.5 M Tris-HCl, pH 8.8)

36.3 gm Tris base was dissolved in about 100 ml distilled water and pH 8.8 was adjusted with 1 M HCl. Finally completed to 200 ml.

(B) Stock Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8)

12.1 gm Tris base was dissolved in about 100 ml distilled water and pH 6.8 was adjusted with 1 M HCl. Finally completed to 200 ml.

(C) Stock Gel Solution (Acrylamide-BIS, 30 % A, 2.67 % C)

60.0 gm acrylamide was dissolved in about 175 ml distilled water and then 1.6 gm BIS (Bis-acrylamide) were added and solution was completed to 200 ml with distilled water. Finally, the solution was filtered through course filter paper.

Note: % A represents acrylamide monomer percent concentration and % C indicates the crosslinking monomer concentration, which were calculated as below:

% A = [(gm acrylamide/total volume)] X 100

% C = [gm BIS/(gm acrylamide + gm BIS)] X 100

(D) 10 % SDS Solution

10 gm SDS was dissolved in water with gentle stirring and completed to a final volume of 100 ml.

(E) Catalyst (10 % Ammonium Persulfate "APS")

Prepared freshly by dissolving 100 mg ammonium persulfate in a final volume of 1 ml distilled water.

(F) Tracking Dye (0.05 % Bromophenol Blue)

Tracking dye solution was prepared by dissolving 5 mg solid bromophenol blue in a final volume of 10 ml.

(G) 5 X Electrode (Running) Buffer (25 mM Tris, 192 mM Glycine, pH 8.3)

Stock running buffer solution was prepared by dissolving and completing 15 gm Tris base, 72 gm glycine to 1 liter distilled water. The pH of the buffer was not adjusted with acid or with base. This buffer was diluted five times and 1 gm solid SDS was added to 1 liter of buffer before use.

(H) 4 X Sample Dilution Buffer (SDS Reducing Buffer)

0.25 M Tris-HCl buffer, pH 6.8 containing 8 % SDS, 40 % glycerol, 20 % 2-mercaptoethanol, 0.004 % bromophenol blue. It was prepared by mixing the following volumes of given solutions :

2.5 ml	1 M Tris-HCl, pH 6.8
4.0 ml	Glycerol
2.0 ml	2-mercaptoethanol
0.4 ml	Tracking Dye

0.8 gm SDS

Distilled water to 10.0 ml

2.2.4.2 Electrophoresis Procedure

Vertical slab gel electrophoresis was carried out using the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A.) that can be used to run two gels simultaneously. The assembly of the glass plate cassettes (8.3 X 7.4 cm) and the process of gel casting were done according to instruction manual provided with the apparatus. Once the cassettes were properly assembled and mounted, the preparation of the separating and stacking gels was started.

The 12 % or 15 % separating gel and 4 % stacking gel polymerizing solutions were prepared just before use by mixing the given volumes of stock solutions in the written order as given in Table 8. The separating gel solution was first prepared with the TEMED added just before casting the gel into the glass assembly from the edge of one of the spacers until the desired height of the solution (about 5 cm) was obtained. Then, the liquid gel was overlaid with distilled water (about 0.5 ml), without disturbing the gel surface, to obtain an even interface between the separating gel and the stacking gel. The gel was then allowed to polymerize at room temperature for a minimum of 30 minutes. After polymerization, the layer of water was removed completely using filter paper without hurting the gel surface. The stacking gel was then poured on the top of the resolving gel and the comb was inserted into the layer of the stacking gel without trapping air bubbles under the teeth of the comb. The gel was then allowed to polymerize for a minimum of 30 minutes. After the gel was polymerized, the comb was removed carefully and the wells were washed with distilled water and filled with electrode buffer. At this point, the gel cassettes were removed from the casting stand, mounted and clamped onto the running frame with the notched glass plate of each cassette facing inside. When running only one gel, the blank plastic plate, provided with the system, was mounted in the place of the second cassette in the casting stand and in the running frame.

Aliquots from the protein samples to be analyzed and from the standards mixture were diluted 3:1 with the 4X sample buffer (3 parts sample and 1 part sample buffer), to have the samples in the final concentrations of 62.5 mM Tris-HCl buffer, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue. Then the samples and standards were placed in a boiling water bath for 5 minutes. Afterwards, protein samples and molecular weight standards (5 – 25 µl) were loaded into different wells using a 25 µl Hamilton syringe with a tipped needle.

Table 8. Formulations for SDS-PAGE separating and stacking gels

	Separating Gel		Stacking Gel
Monomer Concentration	**12 %**	**15 %**	**4 %**
Acrylamide/bis	12.0 ml	15.0 ml	1.3 ml
Distilled water	10.0 ml	7.0 ml	6.1 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	----
0.5 M Tris-HCl, pH 6.8	----	----	2.5 ml
10% (w/v) SDS	300 µl	300 µl	100 µl
10 % APS	185 µl	185 µl	50 µl
TEMED	15 µl	15 µl	10 µl
Total monomer	30 ml	30 ml	10 ml

After loading the samples, the running buffer (135 ml) was added to the compartment formed by the running frame and the cassettes (the upper buffer compartment) and the system was checked for leakage. The running buffer (250ml) was then also added to the outer tank (the lower buffer compartment). Thereafter, the running frame was inserted into the outer tank, the safety cover was replaced and the leads were plugged into the EC250-90 electrophoresis power supply. The power supply was adjusted to give a constant current of 15 mA when the samples were in the stacking gel and 30 mA when the samples passed to the separating gel. Under these conditions the voltage was about 50 V at the beginning and elevated up to 150 V at the end of the run that took a total of about 1.5 hours.

The power supply was switched off, when the dye front is just 0.5 cm from the lower end of the glass plates, the running frame was taken out and the buffer was removed from the upper buffer compartment. Afterwards, the clamps were detached and the cassettes were removed from the running frame. To gain access to the gels in the cassette, the glass plates were pried apart using a spatula taking care not to chip

the edges of the glass plates. The left-top corner of each gel was cut to indicate the order of wells. The gels, usually adhered to one of the glass plates, were taken carefully using gloves and placed in the previously prepared appropriate solutions to stain the samples which have been resolved on the gels, or to prepare the gels for subsequent blotting.

2.2.4.3 Coomassie Blue Staining of the SDS-PAGE Gels

The gel, to be stained, was incubated in the staining solution that contains 0.25 % Coomassie Brilliant Blue R, 50 % methanol and 7 % acetic acid for one hour at room temperature. The gel was then destained by placing in a solution containing 30 % methanol that was changed several times. The complete destaining lasted for approximately 24 hours (Weber and Osborn, 1969). Finally, the destained gel was stored in 7 % acetic acid solution at 4°C.

The relative mobility (R_f) of each protein was determined by dividing its migration distance from the top of the separating gel to the center of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel.

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

The R_f values (abscissa) were plotted against the known molecular weights (logarithmic scale ordinate) and standard line was drawn and its slope was used in the calculation of the molecular weight of proteins.

2.2.4.4 Electroblotting from SDS-PAGE Gels

Electroblotting was carried out using EC140 Mini Blot Module of the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A.), and Polyvinylidene difluoride (PVDF) was used as a blotting membrane. The gels obtained from the SDS-PAGE were electroblotted directly without staining.

Prior to electroblotting, the gels taken from SDS-PAGE were placed for 30 min, with shaking, in the Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol) with shaking (Towbin *et al.*, 1979).

While the gels were incubated in the transfer buffer, the other system components and the transfer membrane were prepared. All of the electroblotting procedure was carried out wearing gloves. The PVDF transfer membrane, with the dimensions of the gel to be transferred, was soaked in 100 % methanol for 30 seconds with shaking, to overcome the hydrophobicity of the membrane. Then, the wet membrane is washed several times with distilled water and then with transfer buffer until it was equilibrated (submerged into the solution and not floating any more), the point at which the membrane is ready to bind the proteins in any blotting application. The membrane should not be allowed to dry, otherwise proteins will not bind to it, so if it does dry during the procedure, the wetting procedure should be repeated again. Afterwards, two pieces of filter paper, the Scotch Brite sponge pads, and the transfer membrane were soaked in the transfer buffer for 15 min with continuous shaking.

The blotting stack was assembled on the top of stainless steel grid cathode located in the trough of the frame stand of the Mini Blot Module, to which a small amount of transfer buffer was added. The configuration of the assembly was as follows:

Top	Cover with Palladium Wire Anode
	Sponge Pad
	Sponge Pad
	Filter Paper
	PVDF Transfer Membrane
	Gel
	Filter Paper
	Sponge Pad
Bottom	Frame stand with Stainless steel Grid Cathode

After the above assembly was prepared, the cover of the electroblotting module was pressed onto the blotting stack and fixed with the clamps after turning assembled blotting module upright and then filled with the transfer buffer (about 100 ml). Thereafter, the fully assembled module was inserted into the outer tank and the safety cover with leads was replaced. The red lead was connected to the anode (+) and the black lead to the cathode (-), were the proteins will be transferred as anions to the direction of anode. The transfer process was performed at room temperature for 50 minutes using a constant voltage of 15 – 20 V. When the blotting was finished, the PVDF membrane was immediately removed and placed in the proper solutions, previously prepared, either for total protein staining or immunostaining.

2.2.4.5 Immunostaining of the PVDF Membranes

Immunostaining was carried out according to the instruction manual provided with the Amplified Alkaline Phosphatase (AP) Western Blotting Kit (Bio-Rad) that was used in the immunostaining of the electroblotted PVDF membranes. All of the incubations were performed in a minimum of 5 ml of solutions in each step with continuous shaking at room temperature.

The electroblotted PVDF membrane was incubated in the blocking solution (5 % non-fat dry milk in Tris buffered saline containing Tween-20 (TTBS) buffer) for 1 to 2 hours. Afterwards, the membrane was incubated with the antibodies diluted in the blocking solution for 1 to 2 hours, where the monoclonal anti-GSTT1-1 (1/10,000 diluted), the polyclonal anti-GST-alpha, mu, pi (1/7,500 diluted) were used. The membrane was then washed five times, each for 5 min with TTBS and incubated with the secondary antibody (biotinylated goat anti-rabbit 1/7,500 diluted in TTBS) for 1 to 2 hours. During the secondary antibody incubation period, the streptavidin - biotinylated AP complex was prepared by the addition of streptavidin to biotinylated AP (both 1/7,500 diluted in TTBS) and allowed to stand at least 1 hour and not more than 3 hours at room temperature. After the incubation with secondary antibody, the membrane was washed again with TTBS (five times, each 5 min) and then incubated for 1-2 hours in the previously prepared streptavidin-

biotinylated AP complex. Afterwards, the membrane was washed again with TTBS (five times, each 5 min) and the AP color developing solution (5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT)) was added. The specific protein bands started to appear after 10 - 30 min. Finally, the membranes were carefully dried and the images were obtained using a scanner connected to the computer or by photography.

2.2.5 Preparation of Permanent Paraffin Sections from Normal and Cancerous Tissues

Tissues were fixed with 10% formalin pH 6.8 for 24 hours at room temperature. Automated tissue processing (Tissue-Tek V.I.P. (Vacuum Infiltration Processor)) was used. Steps of the tissue processing programme are shown in table 9.

Table 9. An overnight schedule for automated tissue processing.

Container	Fluid	Time	Temperature
1	Phophate buffered saline (PBS) pH 7.4	15 min	RT
2	10% Formalin	45 min	RT
3	70% Alcohol	45 min	RT
4	95% Alcohol	1 hr	RT
5	100% Alcohol	1 hr	RT
6	100% Alcohol	1 hr	RT
7	100% Alcohol	1:30 hr	RT
8	100% Alcohol and 1% Cobalt chloride	1:30 hr	RT
9	Xylene	1:30 hr	RT
10	Xylene	1:30 hr	RT
11-12-13	Molten wax (M.P. 56-57°C)	1 hr, 1:30 hr, 1:30 hr	60°C
Total		14 hr, 45 min	

The tissue was then embedded in molten wax according to the required orientation. 4 μm thick sections were cut on a Anglia Scientific Rotary Microtome and mounted on clean, APES coated glass slides (appendix 1). Slides were kept on the incubator at 37°C overnight to ensure good adhesion of the sections.

2.2.6 Haematoxylin and Eosin Staining

The sections were dewaxed in xylene for 5 minutes and rehydrated by immersing into descending concentrations of ethanol (100% ethanol, 95% ethanol, 70% ethanol) each for 2 minutes. After they were rinsed in distilled water for 1-2 minutes, immersed in Harris's Haematoxylin solution for 5 minutes. Then they were rinsed in tap water for 10 seconds, and differentiated in 1% HCl containing 70% alcohol solution (99ml of 70% alcohol and 1ml of concentrated HCl) for 5 seconds. The slides were rinsed in running tap water for 2 minutes and immersed in eosin solution for 5 minutes. After this step, they were rinsed in tap water for 10 seconds, dehydrated in 70% ethanol for 5 seconds, 95% ethanol for 20-30 seconds and 100% ethanol 2 times each for 1 minute, and immersed in xylene for 5 minutes. Finally, the slides were mounted using DPX.

2.2.7 Immunohistochemical Staining for GST alpha, mu, pi, and theta isoenzymes

Immunohistochemical staining of paraffin sections for GST isoenzymes was performed according to the procedures proposed in literature (Forrester et al, 1990; Cairns et al, 1992; Gilbert et al, 1993; Bellamy et al, 1994; Mainwaring et al, 1996; Wrigley et al, 1996) with some modifications. The slides were dewaxed in xylene for 5 minutes and rehydrated in descending concentrations of ethanol (100% ethanol, 95% ethanol, 70% ethanol) each for 2 minutes, and washed in distilled water for 1-2 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 1ml of 30% hydrogen peroxide in 500 ml methanol for 20 minutes at room temperature (RT). The sections were subsequently washed in distilled water for 5

minutes and immersed in 0.005M TBS buffer containing 0.015M sodium chloride, and was performed antigen retrieval for 3 minutes using 0.01M sodium citrate buffer (pH 6.0) (9ml of 0.01M sodium citrate, 41ml of 0.01M citric acid and 450ml distilled water) in a domestic pressure cooker. The sections were subsequently washed in warm water and then transferred in 0.05M TBS for immunohistochemical staining.

Both positive and negative controls were included in each immunohistochemical assay to check that all stages of the reaction are working correctly. Positive controls were sections of known positive material, and was included with each staining run. Normal human liver tissue was used as control for GST-alpha, mu, theta and normal human small intestine tissue was used as control for GST-pi. Breast tissue was used as negative control without primary antibody application. TBS was used instead of the primary antibody.

The slides were stained with avidin-biotin immunoperoxidase technique. Some parameters were altered in order to achieve better staining results concentrations of primary antibody, pressure cooking time for antigen retrieval, and DAB staining periods. Sections were incubated at RT for 30 minutes with either normal swine serum (for anti-GST alpha, mu, pi) (1:50) or normal goat serum (for anti-GST theta) (1:50) diluted in 0.05M TBS to block nonspecific binding. Then primary antibody incubation was performed at 4°C overnight. The dilutions of the primary antibodies were 1:400 for anti-GST alpha, mu, theta and 1:300 for anti-GST pi. The sections were washed in 0.05M TBS 3 times, each for 5 minutes, and then incubated at RT for 1 hour with secondary antibody (swine-anti-rabbit Ig-biotinylated for anti-GST mu, alpha, pi) or (goat-anti-rabbit Ig-biotinylated for anti-GST theta) at a dilution of 1:200. During the secondary antibody incubation period, the streptavidin-biotinylated horseradish peroxidase complex solution (streptABC/HRP) was prepared by the addition of 5ml of 0.05M TBS buffer, 45µl of biotinylated-horseradish peroxidase and 45µl of streptavidin in the written order and allowed to stand at least 20 minutes at 4°C before using. After the incubation with secondary antibody, sections were washed again with TBS (3 times, each 5

minutes) and then incubated with 1-2 drops per slide of pre-mixed strept ABC/ HRP solution for 30 minutes at RT. During streptABC/ HRP solution incubation, DAB substrate solution was prepared by the addition of 20ml of 0.05M TBS buffer, 200ml of 30% hydrogen peroxide solution and 1ml of DAB (10mg/ml) in the written order and allowed to stand at least 10 minutes at 4°C before using. After strept ABC/ HRP solution incubation, the sections were washed again with TBS (3 times, each 5 minutes). After that, they were incubated in pre-mixed DAB substrate solution for 10 minutes at RT and the enzyme reaction was stopped by washing the sections in distilled water for 8 minutes. The slides were counterstained with Harris's haematoxylin solution diluted 1:1 with distilled water at RT for 45 seconds. Afterwards, they were rinsed in distilled water for approximately 1 minute and dehydrated in ascending concentrations of ethanol (70% ethanol, 95% ethanol, 100% ethanol) each for 30 seconds-1 minute and then immersed in xylene for 5 minutes. Finally, they were mounted using DPX.

2.2.8 Observation of Slides and Evaluation

All the sections were examined by a light microscope (Carl Zeiss Jenamed 2 light microscope). Haematoxylin/eosin (HE) stained slides were used to observe the presence of infiltrating carcinoma, intraductal (in situ) carcinoma and normal glandular breast tissue. The blocks which contained only breast stroma, fat, (fibrous tissue) were discarded from the study. In addition, HE stained slides were used for grading of the tumour. Modified Bloom Richardson grading system (appendix 2) (Elston, C. W., 1987) was used for grading. If tumoral tissue was present in more than one paraffin block for one case, total tumour tissue available was taken into consideration for the determination of the grade.

Immunohistochemically stained sections were evaluated without knowledge of the clinical data for each patient. In the evaluation of the immunohistochemical staining, only epithelial cells were taken into consideration. Staining in the stromal cells were noted if present but not further evaluated. In each slide, nuclear and

cytoplasmic staining in the epithelial cells were evaluated separately. Dark-brown colour in cytoplasm and/or nucleus of the epithelial cells was considered as positive staining. Staining intensity and percentage of positively stained cells were graded separately in a scale from 1 to 3. Staining intensity was graded as 0 if no staining was observed, as 1 if weak staining was present, as 2 if moderate staining was observed and as 3 if strong staining was present. Percentage of stained cells was evaluated in a given field. It was graded as 0 if there was not any positively stained cell, as 1 if 1 to 50% of cells was positively stained, as 2 if 51 to 70% of cells was stained and finally 3 if 71 to 100% of cells was stained. In the sections that contained both tumorous and the normal breast parenchyma, two compartments were evaluated individually.

For each slide, for each compartment (normal, in situ, tumour) a cytoplasmic and a nuclear staining score were obtained, each changing from 2 to 6 by adding intensity score and the score obtained by evaluating percentage of positively stained cells. In addition, a total score changing from 4 to 12 was obtained for each compartment by adding cytoplasmic and nuclear staining scores.

For alpha, mu, pi and theta, cytoplasmic, nuclear and total staining scores were compared by correlation analysis in normal, in situ and invasive tumour areas. For each antibody, normal breast tissue, in situ and invasive carcinoma areas were compared by correlation analysis using cytoplasmic, nuclear and total staining scores. Correlations between staining scores of the four antibodies were investigated in normal, in situ, invasive tumour areas.

Staining scores (cytoplasmic, nuclear, and total) in tumour tissue and normal areas were compared for each antibody. The relationship between GST isoenzymes expression and the clinicopathological data were also examined by the chi-square test. Age, chemotherapy and hormonal therapy history, ER status, presence of microcalcification in mammogram, smoking history, menopausal status, tumour stage, and parity were used as clinicopathological parameters for this study.

CHAPTER III

RESULTS

3.1 Evaluation of the HE Stained Slides

A section from each block stained with HE was examined to identify the structure and cell types in normal and tumoural tissue, to determine the tumour type. Figure 4 shows normal breast tissue. It illustrates groups of acini and ductules. Figure 5 shows an infiltrating duct carcinoma of breast (grade II). It illustrates the invasion of breast stroma by nests and cords of tumour cells.

150 sections from 50 patients were evaluated. In 43 of these sections only stromal, fat and fibrous tissue was observed. These sections were discarded from the study because of the absence of breast parenchyma. Remaining 107 blocks were included in the study for further evaluation. 2 (1 and 28) cases were discarded from this study because no breast parenchyma or tumour was observed in any of the three samples obtained from these two cases. The remaining 107 blocks from 48 patients (table 10) was included in the study.

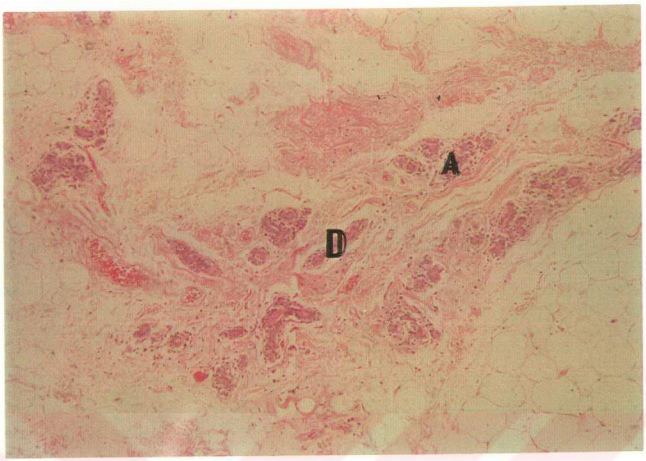


Figure 4 Normal breast tissue (HE x 10). Acini (A), ductules (D).

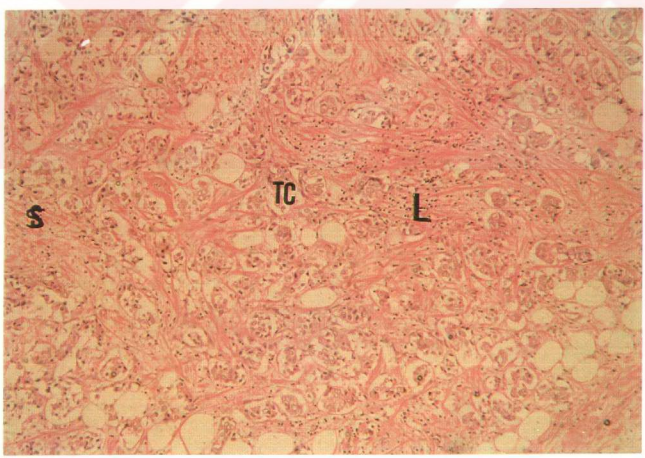


Figure 5 Moderately differentiated (grade II) infiltrating duct carcinoma of breast tissue (HE x 10). Tumour cells (TC), lymphocytes (L), stroma (S).

Table 10. Contents of paraffin blocks from 48 patients.

Patient Number, %		Block 1	Block 2	Block 3
1	2	N	--	--
1	2	--	N	--
4	8	--	T	--
19	40	--	T	T
3	6	T	T	T
1	2	T	--	T
1	2	N	T	--
4	8	--	T	N
4	8	N	T	N
9	20	N	T	T

(N) Normal tissue, (T) Tumour tissue, (--) no parenchyma, only stromal tissue

There are 2 patients who have only normal tissue, 27 patients who have only tumour tissue, 18 patients who have both normal and tumour tissues (table 10). In 48 patients, one or more block representing tumour tissue was available and in 43 of 48 cases, there was in situ carcinoma.

Histologic grade of the tumours was determined on HE stained slides. 5 cases were evaluated as grade I; 36 cases were evaluated as grade II; and 2 cases were evaluated as grade III.

Clinical information for 43 patients who have tumour tisues is shown in table 11. As shown in table 12, 21% of the patients were less than 50 years and 79% of the patients were more than 50 years. 15% of the patients were not given chemotherapy and 85% were given chemotherapy prior to operation. 24% patients

were not given hormonal therapy and 76% were given hormonal therapy. 44% of the patients were ER (-) and 56% were ER (+). 5% of the patients were not showing microcalcification in mammography and 95% of the patients were showing microcalcifications. 39% of the patients were nonsmokers and 61% were smokers. 7% of the patients were premenopausal and 93% were postmenopausal. 19% of the patients were in stage 2 and 3, and 81% were in stage 4. 28% of the patients did not have any children, 26% had one child and 46% have two or more children.

Table 11. Patient details

No. patients	Parity	Age	Meno pause status	T Stage	Chemo therapy	Hormo ne Therap y	Receptor status	Smoking	Micro Calcifica Tion
1	1	>50	Pre	III	NK	NK	NR	NK	P
2	5	<50	Post	IV	T	T	--	NK	P
3	0	<50	Post	IV	T	U	NR	S	P
4	2	>50	Post	II	NK	T	NR	N	P
5	0	>50	Post	IV	T	U	NR	S	P
6	4	>50	Post	III	T	U	+	NK	P
7	0	>50	Post	IV	T	U	NR	N	P
8	0	>50	Post	II	T	T	+	S	P
9	2	>50	Post	IV	U	T	NR	N	P
10	1	>50	Post	IV	T	T	NR	S	P
11	1	>50	Post	IV	T	T	+	S	P
12	3	>50	Post	IV	T	T	-	S	N
13	2	>50	Post	IV	T	T	+	N	P
14	0	<50	Post	II	T	T	NR	N	P
15	5	>50	Post	IV	U	U	NR	N	P
16	0	>50	Post	IV	T	U	-	S	P
17	1	>50	Post	IV	T	T	-	S	P
18	1	>50	Post	IV	T	T	+	S	P
19	1	>50	Post	II	T	T	+	N	P
20	3	<50	Post	IV	T	T	NR	S	P
21	2	>50	Post	IV	T	T	NR	S	P
22	2	>50	Post	IV	T	T	NR	N	P
23	1	>50	Post	IV	T	T	-	S	P
24	4	<50	Post	IV	T	T	-	N	P
25	2	<50	Pre	IV	T	U	NR	N	P
26	3	>50	Post	IV	U	T	+	S	P
27	3	>50	Post	IV	U	T	NR	S	N
28	1	>50	Post	IV	T	T	NR	NK	P
29	0	>50	Post	IV	T	T	+	S	P
30	0	>50	Post	IV	T	T	NR	S	P
31	0	<50	Post	IV	U	T	NR	N	P
32	2	>50	Post	IV	T	T	NR	N	P
33	1	>50	Post	II	T	T	NR	S	P
34	3	>50	Post	IV	T	T	-	S	P
35	1	>50	Post	IV	T	U	+	NK	P
36	0	>50	Post	IV	T	U	+	S	P
37	4	>50	Post	IV	T	T	NR	S	P
38	2	<50	Post	IV	T	T	NR	N	NR
39	2	<50	Post	IV	U	T	NR	N	P
40	1	<50	Pre	III	T	T	NR	S	P
41	0	>50	Post	IV	T	U	NR	N	P
42	0	>50	Post	IV	T	T	NR	S	P
43	3	>50	Post	IV	T	T	-	S	P

NK not known, NR not reported

3.2 Immunohistochemical Study

In general, there was little variation in the GST staining intensity of individual tumour cells and they showed diffuse cytoplasmic staining with weak, moderate, or strong intensities. On the other hand usually stronger staining was observed in normal epithelial cells when compared with tumour tissue. Figure 6 shows that strongly stained normal ductal cells are present within moderately stained tumour cells. There is a great heterogeneity in nuclear staining intensity among normal duct and tumour cells. Figure 7 shows heterogenous nuclear staining intensity from strong to weak for GST alpha in normal duct and tumour cells in the section.

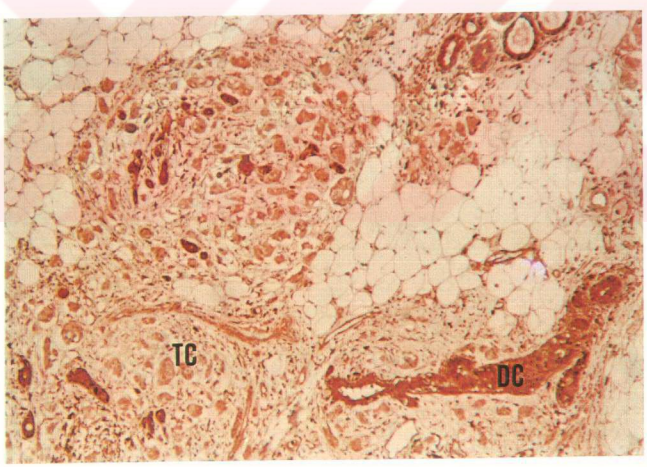


Figure 6 Infiltrating duct carcinoma. There is a diffuse staining tumour cells and normal duct epithelial cells with heterogenous intensities. Cytoplasmic score of normal ductal epithelial cells (NDC) was 6, cytoplasmic score of tumour cells (TC) was 4 (GST theta, x 10).

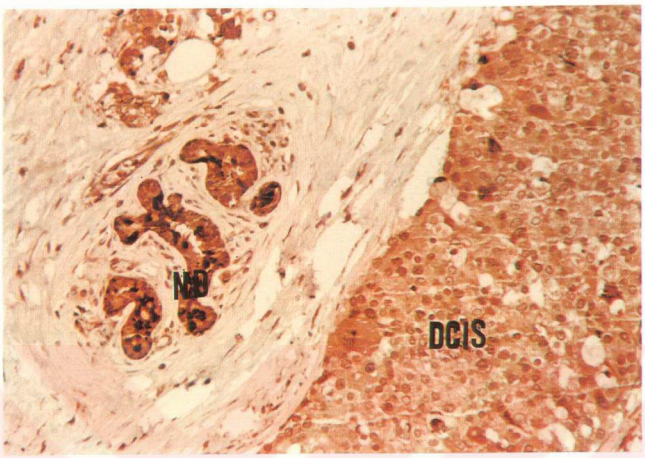


Figure 7 Normal duct (ND) and ductal carcinoma in situ (DCIS). Nuclear staining score of normal ductal epithelial cells was 5 and nuclear score of in situ carcinoma cells was 5 (GST alpha, x 20).

3.2.1 Immunohistochemical Staining for GST pi

In general, there was diffuse cytoplasmic staining of tumour and normal epithelial cells, with weak, moderate or strong positivity. Nuclear staining was usually patchy and different percentage of cells stained positively with varying intensities for the GST pi antibody. GST pi was also detected in nonepithelial cells (eg. fibroblasts and inflammatory cells) to a variable extent. GST pi expression by normal mammary epithelium, stroma, and fat cells is shown in figure 8. Strong positive cytoplasmic and nuclear staining and positively stained breast stroma and fat cells are shown. Arrows show a terminal ductal-lobular unit consisting of intralobular duct and acini and interlobular duct. The staining for GST pi in normal breast tissue was predominantly cytoplasmic with additional nuclear staining in some cells (Figure 8).

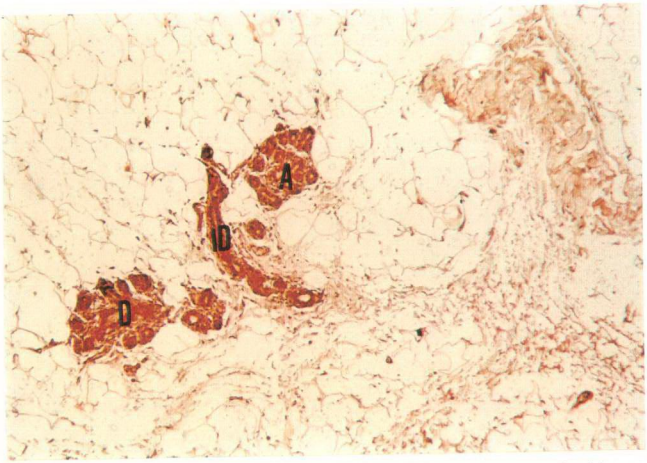


Figure 8 Normal breast tissue. Cytoplasmic staining score was 6 in this case (GST pi x 10). A terminal ductal lobular unit (arrow), intralobular duct (D), interlobular duct (ID), acini (A).

GST pi immunoreactivity in infiltrating ductal carcinoma of breast is shown in figures 9 and 10. An infiltrating ductal carcinoma Grade III (figure 9) shows strong GST pi immunoreactivity and Grade II (figure 10) shows weakly stained tumour cells.

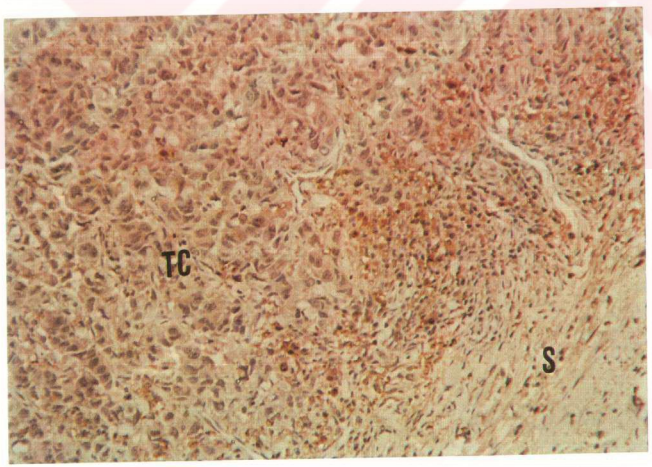


Figure 9 Infiltrating duct carcinoma, grade III. Cytoplasmic staining score of tumour cells was 4 and their nuclear staining score was 2 in this case (GST pi X 20). Stroma (S), tumour cells (TC).

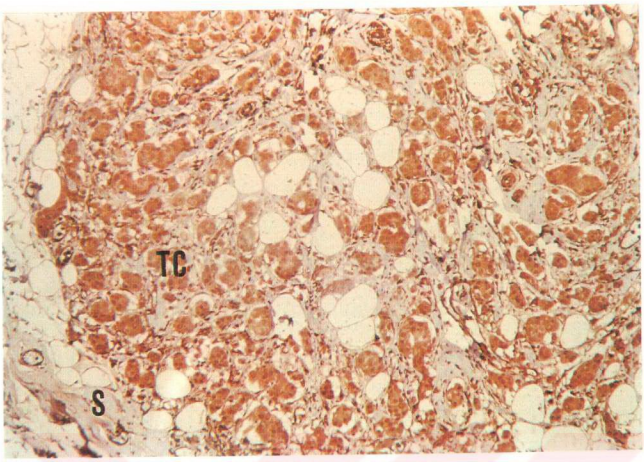


Figure 10. Infiltrating duct carcinoma, grade II. Cytoplasmic staining score was 5 and nuclear staining score was 5 in this case (GST pi X 20). Stroma (S), tumour cells (TC).

3.2.2 Immunohistochemical Staining for GST alpha

In general, expression of GST alpha in epithelial cells was observed in the sections taken from normal and tumour tissues and this was diffuse cytoplamic staining. Nuclear staining was usually patchy and different percentage of cells stained positively with varying intensities for the GST alpha antibody. There was a staining of non-epithelial cells (eg. fibroblasts, inflammatory cells). Immunohistochemical staining for GST alpha in normal breast tissue is shown in figure 11. There was strong positive cytoplasmic and nuclear staining in ductal epithelial cells (figure 11).

An infiltrating ductal carcinoma (grade III) shows moderately stained tumour cells (figure 12). Figure 13 shows moderately stained tumour cells in an infiltrating duct carcinoma (grade II). Normal ductal cells stained stronger than tumour cells in this case (figure 13). Figure 14 shows the distribution of GST alpha in normal duct and in situ duct carcinoma with weak cytoplasmic staining and negative nuclear staining of ductal cells and positive cytoplasmic and nuclear staining in an in situ carcinoma of breast tissue at the same section.

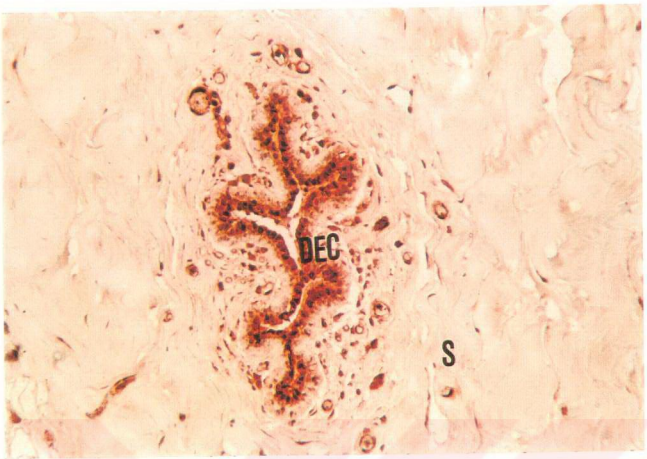


Figure 11. Normal breast tissue. Cytoplasmic score was 5 and nuclear score was 6 in this case (GST alpha x 20). Duct epithelial cells (DEC), stroma (S).

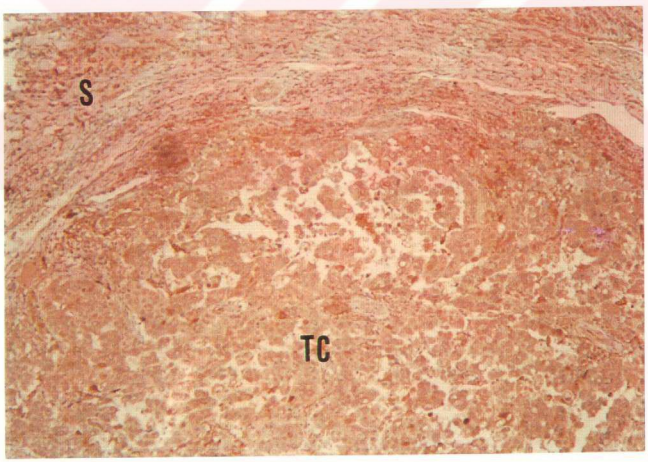


Figure 12. Infiltrating duct carcinoma with weakly stained tumour cells (TC).poorly differentiated (grade III). Cytoplasmic staining score was 5 and nuclear staining score was 3 in this case (GST alpha, x 10). Stroma (S).

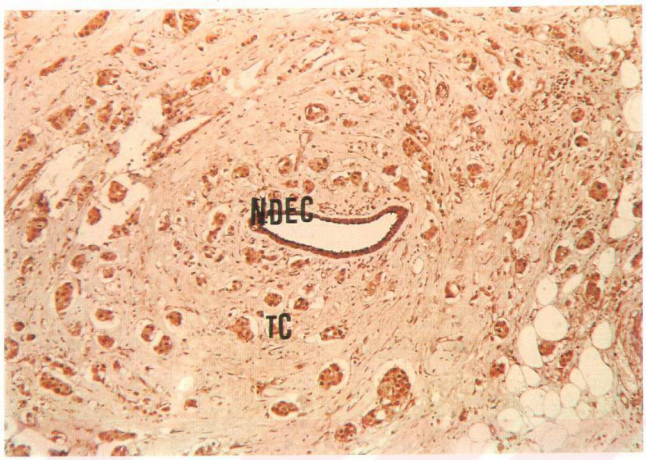


Figure 13. Infiltrating duct carcinoma, moderately differentiated (grade II). Cytoplasmic score was 5 and nuclear score was 6 in this case (GST alpha, x 20). Normal ductal epithelial cells (NDEC), tumour cells (TC).

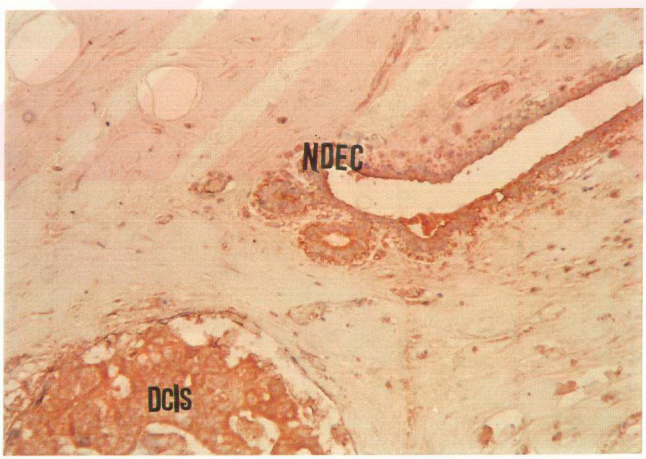


Figure 14. Normal ductal epithelial cells (NDEC) and ductal carcinoma in situ, DCIS) with weak cytoplasmic staining and negative nuclear staining of epithelial cells (GST alpha x 20).

3.2.3 Immunohistochemical Staining for GST mu

In general, there was diffuse cytoplasmic staining of epithelial cells with weak, moderate, or strong positivity. Nuclear staining was usually patchy and different percentage of cells stained positively with varying intensities for the GST mu antibody. GST mu was also detected in non-epithelial cells (eg. fibroblast, inflammatory cells). The staining for GST mu was predominantly cytoplasmic with additional nuclear staining in normal ductal cells in this case (figure 15).

GST mu immunoreactivity in an infiltrating duct carcinoma, in situ duct carcinoma is shown in figure 16 and 17. Figure 16 shows moderate diffuse staining in solid tumour cell nests and in an infiltrating duct carcinoma (grade II). Figure 17 shows moderate GST mu immunoreactivity in tumour cells in in situ duct carcinoma and infiltrating duct carcinoma. Figure 18 shows the distribution of GST mu in an in situ duct carcinoma with weak positive cytoplasmic staining and negative nuclear staining of tumour cells.

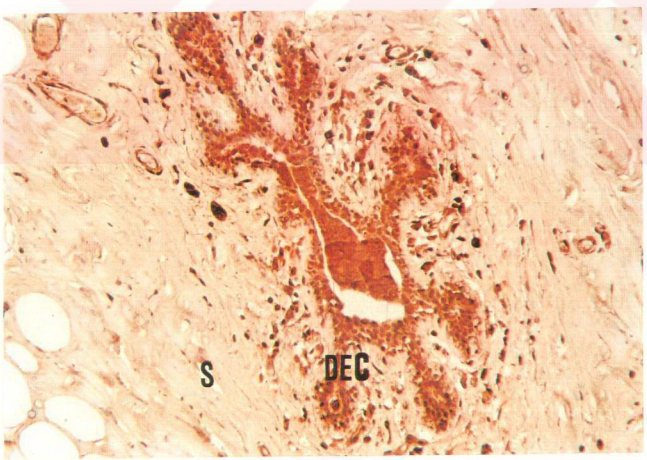


Figure 15. Normal breast tissue. Cytoplasmic score was 5 and nuclear score was 6 in this case (GST mu x 20). Duct epithelial cells (DEC), stroma (S).

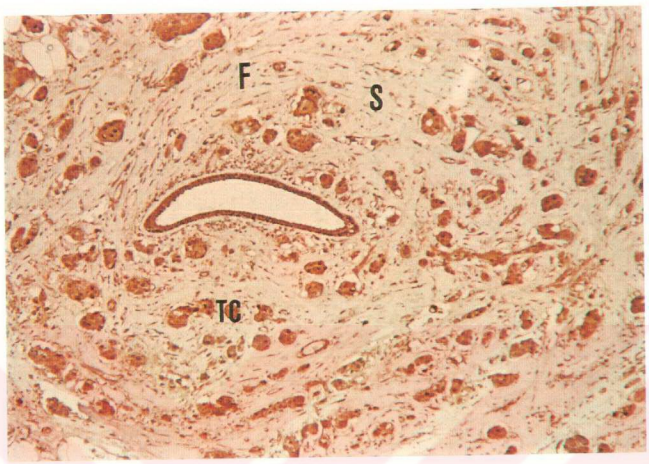


Figure 16. Infiltrating duct carcinoma, moderately differentiated (grade II) (GST mu x 10). Tumour cells (TC), stroma (S), fibroblast (F).

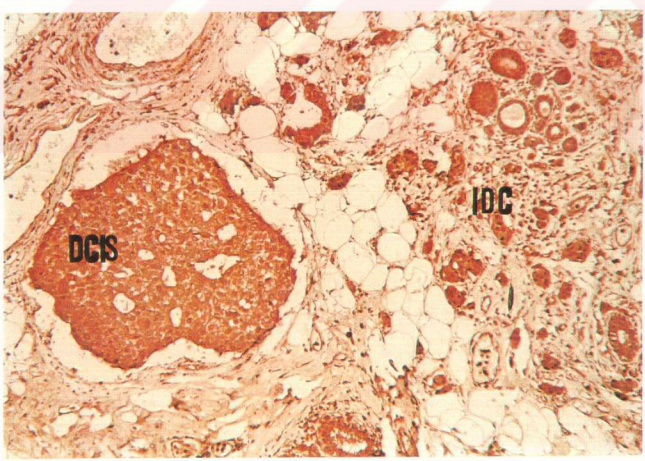


Figure 17. Infiltrating duct carcinoma (IDC), moderately differentiated (grade II) and ductal carcinoma in situ (DCIS) (GST mu x 10). Stroma (S).

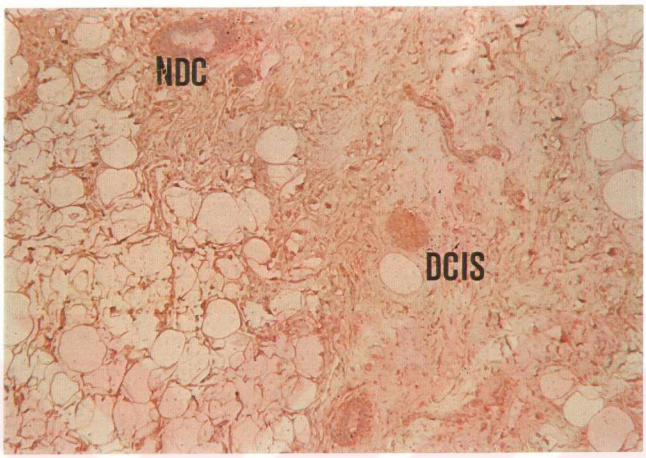


Figure 18. Weak positive cytoplasmic and negative nuclear GST mu staining in normal ductal (NDC) and ductal carcinoma in situ epithelial cells (DCIS) (GST mu, x 10).

3.2.4 Immunohistochemical staining for GST theta

In general, there was diffuse cytoplasmic staining of epithelial cells with weak, moderate or strong positivity in the sections taken from normal and tumour tissues. Nuclear staining was usually patchy and different percentage of cells stained positively with varying intensities for the GST theta antibody. GST theta was also detected in nonepithelial cells to a variable extent. GST theta expression in a normal breast tissue is shown in figure 19. There is strong diffuse cytoplasmic and nuclear immunostaining in all epithelial cells of duct and acini (arrows) in normal breast tissue.

GST theta immunoreactivity in an infiltrating ductal carcinoma and in situ carcinoma is shown in figure 20. It shows moderate staining in tumour epithelial cells in an infiltrating and in situ ductal carcinoma.

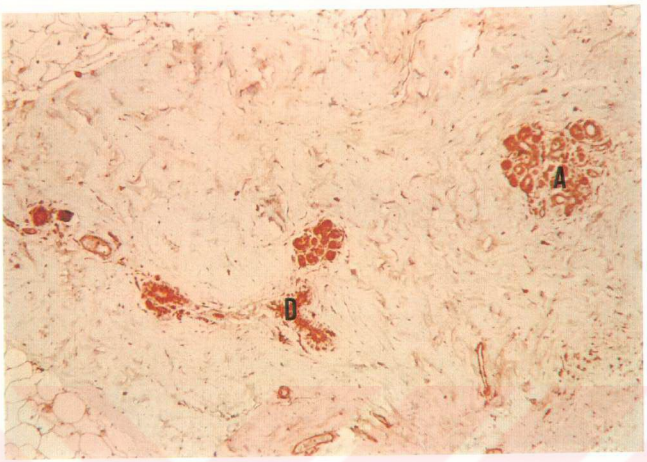


Figure 19. Normal breast tissue (GST theta, x 10). Acini (A), Duct (D).

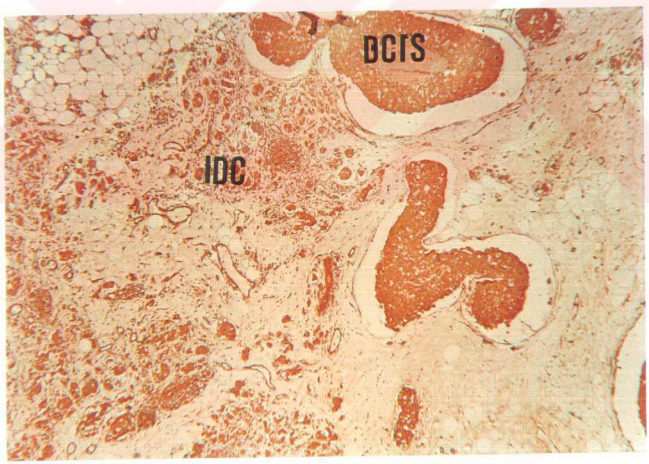


Figure 20. Infiltrating ductal carcinoma (IDC), moderately differentiated (grade II) and ductal carcinoma in situ (DCIS) of breast tissue. Cytoplasmic staining score was 5 in this case (GST theta, x 4).

3.3 Statistical Analysis of Immunohistochemical Data

Epithelial cells (EC), either malignant or normal, showed a diffuse cytoplasmic staining pattern with variable intensities for all of the GST antibodies. Nuclear staining was usually patchy and different percentage of cells stained positively with varying intensities for the GST antibodies.

Statistical analysis showed that there is a strong correlation between nuclear, cytoplasmic and total staining score for alpha, mu, pi and theta antibodies in normal, in situ and invasive tumour areas.

When cytoplasmic, nuclear and total staining scores for different tissue areas (e.g. normal, in situ, invasive tumour) of the same patient was investigated, a strong correlation between staining scores of intraductal (in situ) tumour areas and invasive tumour areas for GST pi, alpha and theta. For GST mu, only cytoplasmic staining score showed a correlation between intraductal (in situ) tumour areas and invasive tumour areas.

Immunohistochemical staining characteristics for GST isoenzymes are summarized in tables 12, 13, 14, 15. Data from 18 patients who have paraffin blocks representing both the tumour and normal breast tissues were included in this statistical analysis (Tables 12, 13, 14, 15). Nuclear GST alpha expression was dependent on the tissue type (normal, in situ, invasive tumour). Cytoplasmic and total staining scores for GST pi were dependent on tissue type. Total staining score (TSS)or GST theta was dependent on tissue type. For GST mu no such relationship was observed.

Table 12. Immunohistochemical staining characteristics for GSTalpha

	Cytoplasmic S S			Nuclear S S			Total S S		
	1-4 no	5 no	6 $x^2$3.80 no p 0.43	1-4 no	5 no	6 $x^2$17.65 no p 0.01*	1-10 no	11 no	12 $x^2$4.52 no p 0.33
Normal	2	7	9	3	12	13	4	6	8
In situ	1	12	5	1	0	17	1	11	6
Tumour	1	12	5	1	1	16	2	11	5

Table 13. Immunohistochemical staining characteristics for GSTmu

	Cytoplasmic S S			Nuclear S S			Total S S		
	1-4 no	5 no	6 $x^2$6.61 no p 0.15	1-4 no	5 no	6 $x^2$5.8 no p 0.2	1-10 no	11 no	12 $x^2$1.07 no p 0.89
Normal	5	8	5	7	2	9	9	4	5
In situ	0	13	5	2	7	9	9	4	5
Tumour	2	11	5	5	6	7	11	2	5

Table 14. Immunohistochemical staining characteristics for GSTpi

	Cytoplasmic S S			Nuclear S S			Total S S		
	1-4 no	5 no	6 $x^2$10.09 no p 0.038*	1-4 no	5 no	6 $x^2$3.56 no p 0.46	1-10 no	11 no	12 $x^2$9.92 no p 0.04*
Normal	4	6	8	5	2	11	7	3	8
In situ	5	11	2	5	6	7	11	5	2
Tumour	5	12	1	5	6	7	11	6	1

Table 15. Immunohistochemical staining characteristics for GSTtheta

	Cytoplasmic S S			Nuclear S S			Total S S		
	1-4 no	5 no	6 $x^2$4.69 no p 0.32	1-4 no	5 no	6 $x^2$3.6 no p 0.46	1-10 no	11 no	12 $x^2$9.87 no p 0.04*
Normal	2	8	8	4	6	8	8	4	6
In situ	0	7	11	1	6	11	7	0	11
Tumour	0	7	11	1	6	11	7	0	11

In intraductal and invasive tumour tissue, cytoplasmic, nuclear and total staining score were dependent on the GST isoenzymes. In normal areas, no such relationship was observed (Tables 16, 17, 18).

Table 16. Relation between GST isoenzymes expressions in breast tumour tissue

	Cytoplasmic S S			Nuclear S S			Total S S		
Antibody	1-4 no	5 no	6 $x^2$17.89 no p 0.006*	1-4 no	5 no	6 $x^2$14.62 no p 0.02*	1-10 no	11 no	12 $x^2$31.23 no p 2.29-05*
Alpha	1	12	5	1	1	16	2	11	5
Mu	2	11	5	5	6	7	11	2	5
Pi	5	12	1	5	6	7	11	6	1
Theta	0	7	11	1	6	11	7	0	11

Table 17. Relation between GST isoenzymes expressions in in situ duct carcinoma.

	Cytoplasmic S S			Nuclear S S			Total S S		
Antibody	1-4 no	5 no	6 $x^2$20.69 no p 0.002*	1-4 no	5 no	6 $x^2$16.34 no p 0.01*	1-10 no	11 no	12 $x^2$27.4 no p 0.0001*
Alpha	1	12	5	1	0	17	1	11	6
Mu	0	13	5	2	7	9	9	4	5
Pi	5	11	2	5	6	7	11	5	2
Theta	0	7	11	1	6	11	7	0	11

Table 18. Relation between GST isoenzymes expressions in normal breast tissue

	Cytoplasmic S S			Nuclear S S			Total S S		
Antibody	1-4 no	5 no	6 $x^2$3.65 no p 0.72	1-4 no	5 no	6 $x^2$7.28 no p 0.29	1-10 no	11 no	12 $x^2$4.11 no p 0.66
Alpha	2	7	9	3	2	13	4	6	8
Mu	5	8	5	7	2	9	9	4	5
Pi	4	6	8	5	2	11	7	3	8
Theta	2	8	8	4	6	8	8	4	6

When the relations between clinical parameters and level of expression of GST isoenzymes were investigated, the following results were obtained.

Alpha: (See Table 19)

Cytoplasmic and total staining scores was dependent on tissue type (normal and invasive tumour tissue). Cytoplasmic, nuclear and total staining score were dependent on age. Cytoplasmic staining score (CSS) was dependent on presence or absence of microcalcification. Cytoplasmic and total staining score was dependent on menopausal status.

No relationship was observed between GST alpha expression and smoking, stage, parity and therapeutic groups.

Mu: (See Table 20)

Nuclear staining score (NSS) was dependent on presence or absence of microcalcification. No relationship was observed for GST mu expression and age groups, parity, therapeutic groups, stage, smoking, menopausal status and ER status.

Pi: (See Table 21)

Cytoplasmic and total staining scores was dependent on tissue type. Total staining score was dependent on parity groups. No relationship was observed between GST pi expression and age groups, therapeutic groups, stage, smoking, menopausal status, ER status and microcalcification status.

Theta: (See Table 22)

Total staining scores was dependent on tissue type. Total staining score was dependent on menopausal status. Total staining score was dependent on hormone therapy. No relationship was observed between GST theta expression and age groups, chemotherapeutic groups, stage, smoking, ER status and microcalcification status.

Table 19 Relationship between GST alpha expression and clinicopathological parameters

			CYTOPLASMIC STAINING					NUCLEAR STAINING					TOTAL				
		#Pat.	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p	1--10 (%)	11 (%)	12 (%)	chi-sq	p
Tissue	T	43	2 (5)	35 (81)	6 (14)	12,32	0,002	3 (7)	4 (9)	36 (84)	1,392	0,499	7 (16)	30 (70)	6 (14)	11,08*(	0,004
	N	21	2 (10)	8 (38)	11 (52)			3 (14)	3 (14)	15 (71)			5 (24)	6 (29)	10 (47)		
Age	<= 50	9	1 (11)	5 (56)	3 (33)	5,018*(	0,081	1 (11)	0 (0)	8 (89)	22,85*(	1E-05	1 (11)	3 (33)	5 (56)	10,30*(	0,006
	> 50	34	1 (3)	30 (88)	3 (9)			2 (6)	28 (82)	4 (12)			6 (18)	25 (74)	3 (9)		
Chemo	U	6	0 (0)	5 (83)	1 (17)	0,37	0,831	0 (0)	0 (0)	6 (100)	1,205	0,547	0 (0)	5 (83)	1 (17)	1,206	0,547
	T	35	2 (6)	28 (80)	5 (14)			3 (9)	3 (9)	29 (82)			6 (17)	24 (69)	5 (14)		
Hormon	U	10	1 (10)	8 (80)	1 (10)	0,926	0,629	1 (10)	1 (10)	8 (80)	0,199	0,905	2 (20)	7 (70)	1 (10)	0,473	0,79
	T	32	1 (3)	26 (81)	5 (16)			2 (6)	3 (9)	28 (88)			4 (13)	23 (71)	5 (16)		
Receptor	neg	8	1 (13)	7 (88)	0 (0)	2,88	0,237	2 (25)	0 (0)	6 (75)	4,114	0,128	2 (25)	6 (75)	0 (0)	1,8	0,407
	pos	10	0 (0)	8 (80)	2 (20)			0 (0)	2 (20)	8 (80)			2 (20)	6 (60)	2 (20)		
Micro	neg	2	0 (0)	2 (100)	0 (0)	10,5*	0,005	0 (0)	0 (0)	2 (100)	0,42	0,811	0 (0)	2 (100)	0 (0)	0,84	0,657
	pos	40	2 (5)	5 (13)	33 (83)			3 (8)	4 (10)	33 (83)			7 (18)	28 (69)	5 (13)		
Smoker	non-sm	15	1 (7)	12 (80)	2 (13)	1,636	0,441	1 (7)	1 (7)	13 (87)	0,207	0,902	2 (13)	11 (73)	2 (13)	0,281	0,869
	smoker	23	0 (0)	19 (83)	4 (17)			1 (4)	1 (4)	21 (91)			2 (9)	17 (74)	4 (17)		
Menopause	pre	3	0 (0)	1 (33)	2 (67)	7,487*·	0,024	0 (0)	1 (33)	2 (67)	2,339	0,311	1 (33)	0 (0)	2 (67)	9,248*·	0,01
	post	40	2 (5)	34 (85)	4 (10)			3 (8)	3 (8)	34 (85)			6 (15)	30 (75)	4 (10)		
Stage	2,3	8	0 (0)	7 (88)	1 (13)	0,517	0,772	0 (0)	2 (25)	6 (75)	3,274	0,195	2 (25)	5 (63)	1 (13)	0,548	0,76
	4	35	2 (6)	28 (80)	5 (14)			3 (10)	2 (6)	29 (84)			5 (14)	25 (71)	5 (14)		
Parity	0	12	1 (8)	9 (75)	2 (17)	1,319	0,858	1 (8)	0 (0)	11 (92)	3,249	0,517	1 (8)	9 (75)	2 (17)	1,051	0,902
	1	11	0 (0)	10 (91)	1 (9)			0 (0)	2 (18)	9 (82)			2 (18)	8 (73)	1 (9)		
	2+	20	1 (5)	16 (80)	3 (15)			2 (10)	2 (10)	16 (80)			4 (20)	13 (65)	3 (15)		

Table 20 Relationship between GST mu expression and clinicopathological parameters

			CYTOPLASMIC STAINING					NUCLEAR STAINING					TOTAL				
		#Pat.	1 -- 4	5	6	chi-sq	p	1 -- 4	5	6	chi-sq	p	1 -- 10 (%)	11 (%)	12 (%)	chi-sq	p
Tissue	T	43	5 (12)	28 (65)	10 (23)	2,227	0,328	11 (26)	13 (30)	19 (44)	1,939	0,379	24 (56)	9 (21)	10 (23)	1,329	0,514
	N	21	5 (24)	10 (48)	6 (29)			7 (33)	3 (14)	11 (52)			9 (43)	7 (33)	5 (24)		
Age	<= 50	9	0 (0)	7 (78)	2 (22)	1,609	0,447	2 (22)	2 (22)	5 (56)	0,625	0,732	3 (33)	3 (33)	3 (33)	1,968	0,374
	> 50	34	5 (15)	21 (62)	8 (24)			9 (26)	11 (32)	14 (41)			20 (58)	6 (18)	8 (24)		
Chemo	U	6	0 (0)	3 (50)	3 (50)	2,844	0,241	1 (17)	0 (0)	5 (83)	4,232	0,121	1 (17)	2 (33)	3 (50)	3,552	0,169
	T	35	4 (11)	24 (69)	7 (20)			10 (29)	11 (31)	14 (40)			21 (60)	8 (23)	8 (23)		
Hormon	U	10	0 (0)	9 (90)	1 (10)	3,964	0,138	2 (20)	5 (50)	3 (30)	2,975	0,226	6 (60)	2 (20)	2 (20)	0,35	0,839
	T	32	5 (16)	18 (56)	9 (28)			9 (28)	7 (22)	16 (50)			16 (50)	7 (22)	9 (28)		
Receptor	neg	8	0 (0)	7 (88)	1 (13)	2,948	0,229	3 (38)	2 (25)	3 (38)	1,279	0,527	5 (62)	2 (25)	1 (13)	3,715	0,156
	pos	10	1 (10)	5 (50)	4 (40)			2 (20)	5 (50)	3 (30)			6 (60)	0 (0)	4 (40)		
Micro	neg	2	0 (0)	2 (100)	0 (0)	1,05	0,592	2 (100)	0 (0)	0 (0)	5,91*8	0,052	2 (100)	0 (0)	0 (0)	1,735	0,42
	pos	40	5 (13)	26 (65)	9 (23)			9 (23)	13 (33)	18 (44)			21 (52)	10 (25)	9 (23)		
Smoker	non-smo	15	2 (13)	10 (67)	3 (20)	0,609	0,737	4 (27)	5 (33)	6 (40)	1,471	0,479	8 (53)	3 (20)	4 (27)	0,373	0,83
	smoker	23	2 (9)	14 (61)	7 (30)			6 (26)	4 (17)	13 (57)			10 (44)	6 (26)	7 (30)		
Menopause	pre	3	0 (0)	3 (100)	0 (0)	1,728	0,422	1 (33)	2 (67)	0 (0)	2,917	0,233	2 (67)	0 (0)	1 (33)	0,855	0,652
	post	40	5 (13)	25 (63)	10 (25)			10 (25)	11 (28)	19 (47)			21 (52)	9 (23)	10 (25)		
Stage	2,3	8	2 (25)	6 (75)	0 (0)	3,945	0,139	1 (13)	5 (62)	2 (25)	4,861	0,088	6 (75)	2 (25)	0 (0)	3,442	0,179
	4	35	3 (9)	22 (63)	10 (29)			10 (29)	8 (23)	17 (49)			17 (49)	7 (20)	11 (31)		
Parity	0	12	1 (8)	7 (58)	4 (33)	1,288	0,863	2 (17)	3 (25)	7 (58)	5,23	0,265	5 (42)	3 (25)	4 (33)	2,115	0,715
	1	11	1 (9)	8 (73)	2 (18)			1 (10)	5 (45)	5 (45)			6 (55)	3 (27)	2 (18)		
	2+	20	3 (15)	13 (65)	4 (20)			8 (40)	5 (25)	7 (35)			13 (65)	3 (15)	4 (20)		

Table 21 Relationship between GST pi expression and clinicopathological parameters

			CYTOPLASMIC STAINING					NUCLEAR STAINING					TOTAL				
		#Pat.	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p
Tissue	T	43	10 (23)	32 (75)	1 (2)	17,93	1E-04	9 (20)	17 (40)	17 (40)	4,438	0,109	26 (61)	16 (37)	1 (2)	17,65	1E-04
	N	21	4 (19)	8 (38)	9 (43)			5 (24)	3 (14)	13 (62)			8 (38)	4 (19)	9 (43)		
Age	<= 50	9	2 (22)	7 (78)	0 (0)	0,287	0,866	2 (22)	2 (22)	5 (56)	1,611	0,447	4 (44)	5 (56)	0 (0)	1,777	0,411
	> 50	34	8 (24)	25 (73)	1 (3)			7 (21)	15 (44)	12 (35)			22 (65)	11 (32)	1 (3)		
Chemo	U	6	1 (17)	5 (83)	0 (0)	0,316	0,854	1 (17)	3 (50)	2 (33)	0,211	0,9	4 (67)	2 (33)	0 (0)	0,229	0,892
	T	35	8 (23)	26 (74)	1 (3)			7 (20)	14 (40)	14 (40)			21 (60)	13 (37)	1 (3)		
Hormon	U	10	3 (30)	7 (70)	0 (0)	0,829	0,661	2 (20)	5 (50)	3 (30)	0,656	0,72	7 (70)	3 (30)	0 (0)	0,78	0,677
	T	32	6 (19)	25 (78)	1 (3)			6 (19)	12 (38)	14 (44)			18 (56)	13 (41)	1 (3)		
Receptor	pos	10	1 (10)	9 (90)	0 (0)	NA	NA	3 (30)	3 (30)	4 (40)	0,788	0,675	6 (60)	4 (40)	0 (0)	NA	NA
	neg	8	1 (13)	7 (88)	0 (0)			1 (12)	3 (38)	4 (50)			4 (50)	4 (50)	0 (0)		
Micro	neg	2	0 (0)	2 (100)	0 (0)	0,745	0,689	0 (0)	2 (100)	0 (0)	3,088	0,214	2 (100)	0 (0)	0 (0)	1,292	0,524
	pos	40	10 (25)	29 (73)	1 (3)			9 (22)	15 (38)	16 (40)			24 (60)	15 (38)	1 (3)		
Smoker	non-smo	15	2 (13)	12 (80)	1 (7)	2,279	0,32	2 (13)	5 (33)	8 (54)	0,84	0,657	7 (47)	7 (47)	1 (7)	1,987	0,37
	smoker	23	6 (26)	17 (74)	0 (0)			5 (22)	9 (39)	9 (39)			14 (61)	9 (39)	0 (0)		
Menopause	pre	3	2 (67)	1 (33)	0 (0)	3,42	0,181	2 (67)	0 (0)	1 (33)	4,586	0,101	2 (67)	1 (33)	0 (0)	0,109	0,947
	post	40	8 (20)	31 (78)	1 (3)			7 (18)	18 (45)	15 (38)			24 (60)	15 (38)	1 (3)		
Stage	2,3	8	3 (38)	5 (63)	0 (0)	1,274	0,529	2 (25)	3 (38)	3 (38)	0,098	0,952	5 (62)	3 (38)	0 (0)	0,236	0,889
	4	35	7 (20)	27 (77)	1 (3)			7 (20)	14 (40)	14 (40)			21 (60)	13 (37)	1 (3)		
Parity	0	12	3 (25)	9 (75)	0 (0)	4,067	0,397	3 (25)	4 (33)	5 (42)	3,62	0,46	7 (58)	0 (0)	5 (42)	13,58*(	0,009
	1	11	4 (36)	7 (64)	0 (0)			4 (36)	3 (27)	4 (36)			7 (64)	4 (36)	0 (0)		
	2+	20	2 (10)	17 (85)	1 (5)			2 (10)	10 (50)	8 (40)			12 (60)	7 (35)	1 (5)		

NA not applicable

Table 22 Relationship between GST theta expression and clinicopathological parameters

		CYTOPLASMIC STAINING						NUCLEAR STAINING						TOTAL				
		#Pat.	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p	1 -- 4 (%)	5 (%)	6 (%)	chi-sq	p
Tissue	T	43	1 (2)	13 (30)	29 (68)	3,124	0,21	4 (9)	8 (19)	31 (72)	2,548	0,28	12 (28)	2 (5)	29 (67)	7,31*	0.03*
	N	21	2 (10)	9 (42)	10 (48)			4 (19)	6 (29)	11 (52)			8 (38)	5 (24)	8 (38)		
Age	<= 50	9	0 (0)	4 (44)	5 (56)	1,71	0,425	2 (22)	2 (22)	5 (56)	2,554	0,279	3 (33)	2 (22)	4 (45)	4,952	0,084
	> 50	34	1 (3)	8 (24)	25 (73)			2 (6)	6 (18)	26 (76)			8 (24)	1 (3)	25 (73)		
Chemo	U	6	0 (0)	1 (17)	5 (83)	0,786	0,675	1 (17)	0 (0)	5 (83)	1,873	0,392	1 (17)	0 (0)	5 (83)	0,846	0,655
	T	35	1 (3)	11 (31)	23 (66)			3 (9)	8 (23)	24 (68)			10 (29)	2 (6)	23 (65)		
Hormon	U	10	1 (10)	2 (20)	7 (70)	3,539	0,17	1 (10)	0 (0)	9 (90)	3,137	0,208	1 (10)	2 (20)	7 (70)	8,01*	0.02*
	T	32	0 (0)	10 (31)	22 (69)			3 (9)	8 (25)	21 (66)			11 (34)	0 (0)	21 (66)		
Receptor	pos	10	0 (0)	3 (30)	7 (70)	NA	NA	1 (10)	2 (20)	7 (70)	1,8	0,407	3 (30)	0 (0)	7 (70)	1,878	0,391
	neg	8	0 (0)	2 (25)	6 (75)			1 (13)	0 (0)	7 (87)			1 (13)	1 (13)	6 (74)		
Micro	neg	2	0 (0)	0 (0)	2 (100)	0,941	0,625	0 (0)	0 (0)	2 (100)	0,84	0,657	0 (0)	0 (0)	2 (100)	1,05	0,592
	pos	40	5 (13)	8 (20)	27 (67)			4 (10)	8 (20)	28 (70)			12 (30)	2 (5)	26 (65)		
Smoker	Non-smo	15	0 (0)	5 (33)	10 (67)	0,828	0,661	1 (7)	3 (20)	11 (73)	0,079	0,961	4 (27)	1 (7)	10 (66)	0,14	0,932
	smoker	23	1 (4)	6 (26)	16 (70)			2 (9)	5 (22)	16 (69)			7 (30)	1 (4)	15 (66)		
Menopause	pre	3	0 (0)	2 (67)	1 (33)	2,425	0,297	0 (0)	1 (33)	2 (67)	0,689	0,708	1 (33)	1 (33)	1 (33)	6,29*	0.04*
	post	40	1 (3)	10 (25)	29 (72)			4 (10)	7 (18)	29 (72)			11 (28)	1 (3)	28 (69)		
Stage	2,3	8	0 (0)	2 (25)	6 (75)	0,297	0,862	0 (0)	2 (25)	6 (75)	1,142	0,565	2 (25)	0 (0)	6 (75)	0,57	0,752
	4	35	1 (3)	10 (29)	24 (68)			4 (11)	6 (17)	25 (72)			10 (29)	2 (6)	23 (65)		
Parity	0	12	1 (8)	2 (17)	9 (75)	4,288	0,368	1 (8)	1 (8)	10 (84)	1,495	0,828	2 (17)	1 (8)	9 (75)	2,084	0,72
	1	11	0 (0)	3 (27)	8 (73)			1 (9)	2 (18)	8 (73)			3 (27)	0 (0)	8 (73)		
	2+	20	0 (0)	8 (40)	12 (60)			2 (10)	5 (25)	13 (65)			7 (35)	1 (5)	12 (60)		

NA Not applicable

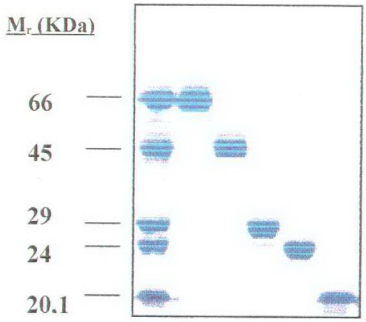
3.4 SDS-PAGE and Western Blotting

Cytosolic fraction from the breast control and tumour were analyzed by SDS-PAGE in order to resolve the subunit composition of GSTs. The molecular weight was calculated from the standard curve for molecular weight markers (Figure 21). The Mr values of the subunits alpha, mu, pi, and theta were respectively 24, 26.700, 26, 25 kDa estimated using molecular marker proteins run on the same gel.

Western blots were carried out on the 21 matched breast control and tumour samples to assess the relative GST isoenzyme content. Representative blots using polyclonal antibodies to GST alpha, mu, pi, and monoclonal antibody to GST theta are shown in Figure 22. All the normal and tumour samples expressed a protein that reacted with the antibody to GST pi. In the samples 16, 19, 3, 4, 8, 20, 21, 10-14, (57%) GST pi content was similar both between the tumours and between normal and tumour tissue from the same patient. However, in six (patient 1, 2, 3, 5, 6, 9, 18 and 17) of 21 samples (38%) evaluated, the breast tumour sample contained more proteins reacting with GST pi antibody than normal breast tissue. In one (15) patient (5%) the normal value is higher as compared to tumour breast tissue. In all normal and tumour specimens however, a low molecular mass band was visible in addition to the 26kDa band of glutathione S-transferase pi.

Proteins reacting with antibodies against the alpha class GST were only present at low levels but were expressed in all the breast normal and tumour samples, except patient 8, despite strongly positive liver control samples. Intriguingly, four of the tumour samples, namely, the patients 6, 4, 20 and 21, (20%) expressed higher levels of an alpha class GST protein than the normal tissue. In the patients 1, 2, 3, 5, 9, 19, 13, 12 and 11 of the samples (45%) evaluated, normal breast samples contained higher levels of an alpha class GST protein, than the tumour samples. The level of this protein was present at similar concentrations in normal and tumour tissue from the ten (18, 17, 16, 15, 10, 14, 7) patients (35%).

(a)



(b)

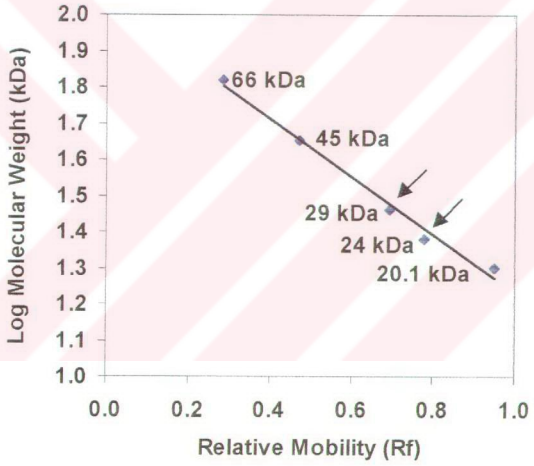


Figure 21. (a) SDS-PAGE (12 %); for the molecular weight markers: - Bovine Albumin (M_r 66,000), Egg Albumin (M_r 45,000), Carbonic Anhydrase (M_r 29,000), Trypsinogen (M_r 24,000), Trypsin Inhibitor (M_r 20,100). **(b)** Typical molecular weight standard curve (12 % SDS-PAGE). The range of the subunit molecular weight of GSTT1-1, GST-mu, GST-alpha, and GST-pi are within the arrows.

All of the breast normal and tumour samples contained detectable levels of GST mu isoenzyme. Patients 1, 2, 4, 10, 11 and 14 (29%) expressed higher levels of a mu class GST protein in tumour than the normal tissue. However, patients 17, 5, 6 and 7 (19%) expressed higher levels of a mu class GST protein than the tumour tissue. The level of this protein was present at similar concentrations in normal and tumour tissue from the patients 13, 8, 3, 9, 12, 15, 16, 18, 19, 20, and 21 (52%).

The levels of alpha- and mu-class GST proteins varied significantly among individuals.

Protein reacted with the antibody to the theta-class GST were only present at very low levels but were expressed in all of the breast samples. The level of this protein was present at similar concentrations in normal and tumour tissue from the patients 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 (71%). In four (1, 3, 7, 21) patients the normal value is higher as compared to tumour breast tissue, whereas in the other (2, 4) patients tumour values are higher (10%).

The classification of samples as positive (+, ++) or negative (-) for each GST isoenzyme class on western blotting and enzyme activities of each sample for CDNB and EPNP was shown in table 23. CDNB is the substrate for GST alpha, mu and pi. EPNP is the substrate for GST theta. All samples contained detectable levels of GST pi, mu, alpha, theta isoenzymes but most of them contained low levels of GST alpha, theta isoenzymes.

Table 23. The classification of the matched breast normal (N) and tumour (T) tissues for each GST isoenzyme class on western blotting and GSTs activities against CDNB and EPNP.

Patient no	GST alpha	GST mu	GST pi	GST theta	CDNB* N/T	EPNP* N/T
1 N	++	+	+	++	22.7/140.7	16.4/44.5
T	+	++	++	+		
2 N	++	+	+	+	24.2/70.5	0.0/0.0
T	+	++	++	++		
3 N	++	+	+	++	6.4/38.2	21.1/22.7
T	+	+	++	+		
4 N	+	+	+	+	38.0/96.2	38.9/47.8
T	++	++	+	++		
5 N	++	++	+	+	47.5/41.6	59.8/69.2
T	+	+	++	+		
6 N	+	++	+	+	30.2/191.1	0.0/0.0
T	++	+	++	+		
7 N	+	++	+	++	18.8/2.1	11.0/32.0
T	+	+	+	+		
8 N	-	+	+	+	67.7/74.7	17.1/18.8
T	-	+	+	+		
9 N	++	+	+	+	48.2/54.8	21.5/25.8
T	+	+	++	+		
10 N	+	+	+	+	43.3/123.6	38.3/54.8
T	+	++	+	+		
11 N	++	+	+	+	57.9/165.3	15.8/21.1
T	+	++	+	+		
12 N	++	+	+	+	115.5/165.3	20.7/24.5
T	+	+	+	+		
13 N	++	+	+	+	131.4/184.4	47.4/57.8
T	+	+	+	+		
14 N	+	+	+	+	197.6/300.1	32.9/23.7
T	+	++	+	+		
15 N	+	+	++	+	102.4/93.0	0.0/0.0
T	+	+	+	+		
16 N	+	+	+	+	49.9/320.8	15.9/45.7
T	+	+	+	+		
17 N	+	++	+	+	293.9/517.7	55.2/73.7
T	+	+	++	+		
18 N	+	+	+	+	112.2/98.1	0.0/0.0
T	+	+	++	+		
19 N	++	+	+	+	220.9/142.0	0.0/0.0
T	+	+	+	+		
20 N	+	+	+	+	74.7/133.8	11.7/27.4
T	++	+	+	+		
21 N	+	++	+	++	66.4/174.5	0.0/0.0
T	++	+	+	+		

*Enzyme activities data are taken from (Ergüney,1999).

As it is clear from Table 23, The GST activities were higher in the tumour cytosolic fractions, against both CDNB and EPNP, in the majority of the 21 patients examined. Case 4, 6, 20, 21 (19%), case 1, 2, 4, 10, 11, 14 (29%), case 1, 2, 3, 5, 6, 9, 18, 17 (38%) and Case 2, 4 (10%) have more GST alpha, mu, pi and theta protein respectively in tumour than normal breast tissue in western blotting analysis. Case 2, 6, 15, 18, 19, 21 (29%) have no GST activity in breast normal and tumour tissues for GST theta but have contained GST theta protein in normal breast and tumour tissues. Remaining patients either similar or higher GST protein in normal than tumour breast tissue (Table 23).

While GSTs activity against CDNB as a substrate was detected in all of the matched 21 cytosolic fraction, 29% of the cytosolic fractions (6 out of 21) had no activity of GSTs against EPNP as a substrate.

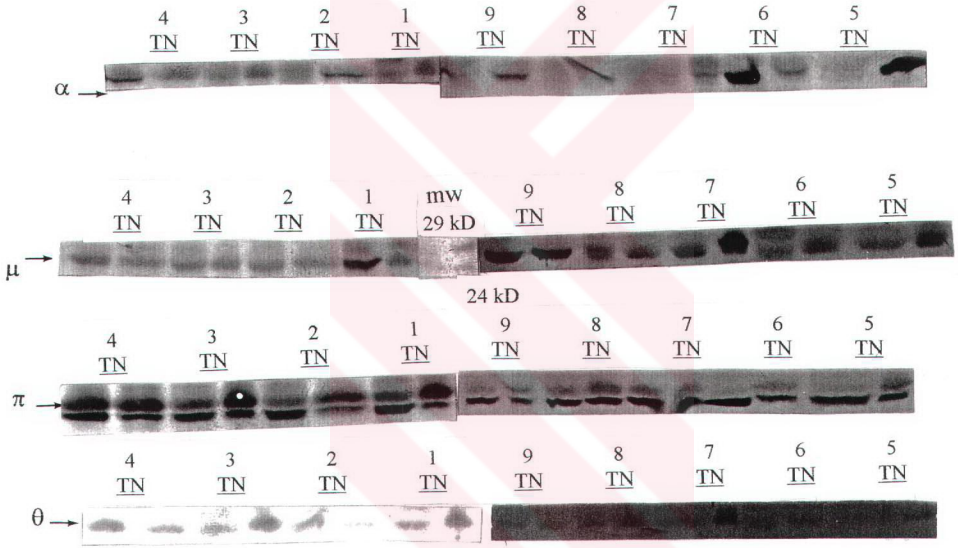


Figure 22. Immunodetection of glutathione S-transferases class alpha, mu, pi and theta in normal breast and corresponding tumour cytosolic fractions (~40μg protein) from breast normal (N) and tumour (T) were subjected to SDS-PAGE (12% acrylamide, w/v) and subsequent western blotting. Blots were incubated with polyclonal antibodies against class alpha (upper panel), class mu (lower middle panel), class pi (upper middle panel) and monoclonal antibody against class theta (lower panel) glutathione S-transferases. Numbers correspond with patient numbers of Table 23. Arrows, positions of the proteins of interest: GST alpha, mu, pi, theta.

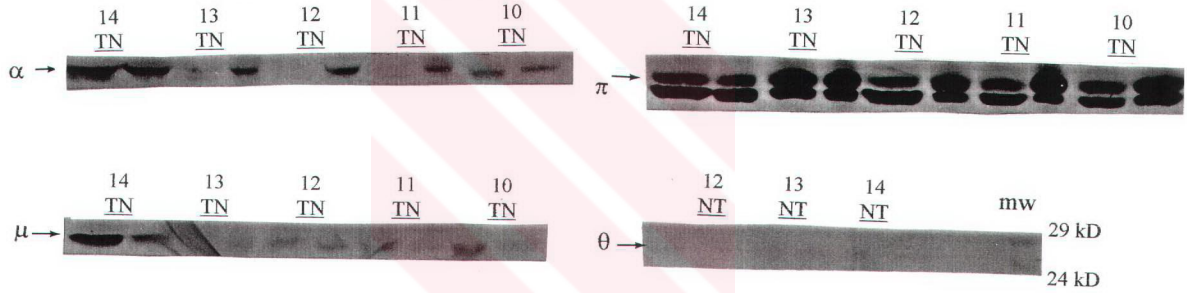


Figure 22 (continued) Immunodetection of glutathione S-transferases class alpha, mu, pi and theta in normal breast and corresponding tumour cytosolic fractions (~40μg protein) from breast normal (N) and tumour (T).

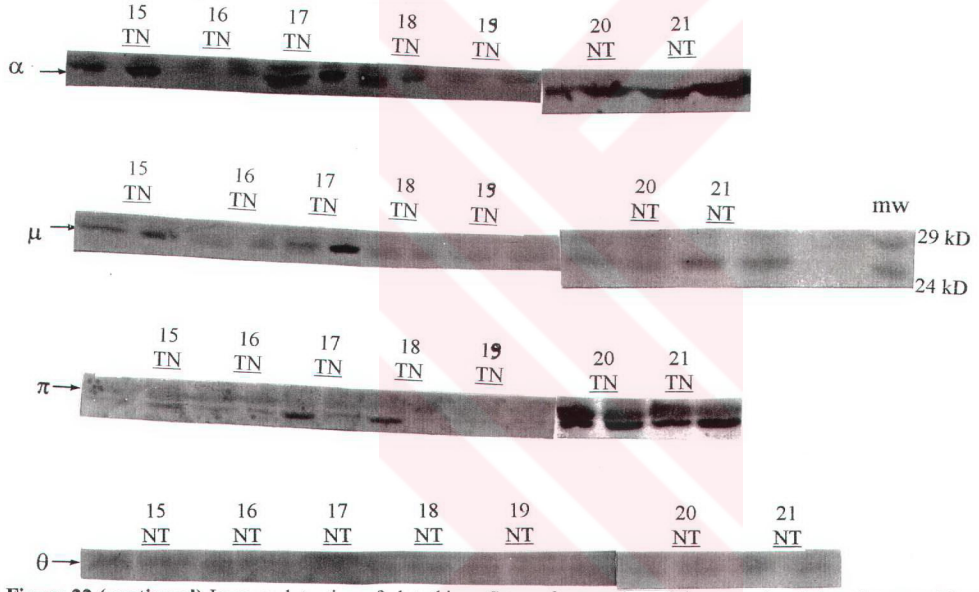


Figure 22 (continued) Immunodetection of glutathione S-transferases class alpha, mu, pi and theta in normal breast and corresponding tumour cytosolic fractions (~40μg protein) from breast normal (N) and tumour (T).

CHAPTER IV

DISCUSSION

It has been suggested that the glutathione S-transferases are the most important class of enzymes in the protection of the cell from the toxic effects of reactive electrophiles (Armstrong, 1991). Research during the last 10 years has clearly established a role for these enzymes in mediating resistance to a wide variety of electrophiles, from the endogenous products of oxidative metabolism to environmental carcinogens and anticancer drugs. A continuing goal is to identify which isoenzymes are important in the detoxification of specific electrophiles, and to determine if it would be possible to regulate the important isoenzymes, using pharmacological agents.

Wide interorgan and interindividual variation of GST isoenzymes has been reported in different tissues (Sherman *et. al.*, 1983; Carrigall *et. al.*, 1988) The strikingly different distribution of the pi, alpha, mu and theta GST in the liver, pancreas, kidney, placenta, breast, colon and other tissues may help to shed new light on the functions of these isoenzymes and may allow additional insight into the interindividual and interorgan differences in susceptibility to tissue damage and carcinogenesis after exposure to certain xenobiotics.

The isoenzymes of GSTs differ in their substrate specificity. Therefore, the presence or absence of particular GST isoenzymes may account for the response or resistance of tumours to particular chemotherapeutic drugs as already established for cultured cell lines. Both increased and reduced expression levels of specific

GST isoenzymes in tumour, particularly those that have become resistant to anti-cancer drugs, has suggested a role for these proteins in the development of resistance to chemotherapy. Determination of the GST isoenzyme profile of a cancer tissue could have prognostic value in the selection of treatment if the levels of expression show a degree of variation before and after administration of chemotherapeutic agents.

As breast cancer is a major cause of morbidity and mortality in women and considering the established role of GST isoenzymes in carcinogenesis and drug detoxification as well as the reported polymorphic expression of hGSTT1-1, it is of special interest to identify the GSTs in breast tumours and normal tissues. This study represents the first comprehensive description of the four classes of GST in normal and tumoural human breast. Other investigators have studied the distribution of alpha, mu, and pi isoenzymes (Cairns et al.,1992; Forrester et al., 1990; Gilbert et al., 1993). In spite of their toxicological significance and important role in carcinogenesis, relatively few reports for the GST theta distribution on human cancer tissue. Class Theta GSTs have never been studied in human tissues by immunohistochemistry except in liver and lung before this study.

We have studied the localization of alpha, pi, mu and theta type GSTs in paraffin-embedded sections of normal breast and breast carcinomas (infiltrating duct carcinoma) using an immunohistochemical technique. Using immunohistochemistry, the specific cell types containing GSTs can be identified and, in particular, tumour cells expressing different GSTs can be specifically recognized. However, biochemical studies of breast tumours have used tissue homogenates which inevitably contain non-neoplastic elements such as stroma and normal breast epithelium. In this study, in many cases, expression was far greater in the accompanying stromal cells and inflammatory infiltrate, and normal mammary epithelium also stained consistently and strongly. This observation emphasize the usefulness of immunohistochemical technique in demonstrating the distribution of enzymes and other proteins in tissues, such as tumours, composed of a variety of cell types.

Immunohistochemical techniques also provide an indication of intracellular localization. In this study, epithelial cells either malignant or non-malignant staining with any of four GST antibodies generally showed a diffuse cytoplasmic staining and patchy nuclear staining. In addition, there is a great heterogeneity in nuclear staining intensity among normal and tumour cells for the GST alpha, mu, pi and theta isoenzymes. Intracellular localisation of the isoenzymes may provide additional information about the functions of the isoenzymes. As GSTs are cytosolic proteins, the significance of nuclear staining is uncertain. McKay et al. (1993), Murray et al. (1993), Hamada et al. (1994), Harrison et al. (1989), Collier et al. (1993), and Shiratori et al. (1987) have observed nuclear staining in colon, breast, ovary, renal, pancreas, uterine cervix carcinoma tissues, in addition to cytoplasmic staining in their studies. However, there was no comment on the significance of nuclear staining in the afformentioned studies. The rat GST YbYb, which is present within rat cell nuclei (Bennet C. F., 1987), is able to bind steroid hormones (Homma H., 1985). Cairns et. al (1992) suggested that, there may be a similar role for GSTs in the human breast, given the importance of steroid hormones as regulators of the function of mammary epithelium. GST theta is known to have peroxidase activity and it is associated with the repair of oxidative DNA damage. This function of GST theta may be an explanation of nuclear localisation. However, relations between the nuclear localisation of GST isoenzymes and their functions remain to be further clarified.

Cairns et. al. (1992) using immunohistochemical staining, detected alpha class GST in 19 per cent of tumour epithelium, mu class GST in 42 per cent of tumours, pi class GST in 47 per cent of tumours. In this study, alpha, mu, pi, and theta class GST isoenzymes were detected in 100 per cent of our cases. Our result was not consistent with them. The reason might be that Cairns et. al. used different commercial source of primary antibodies and different detection system (peroxidase antiperoxidase) in their study compared to our study.

In this study for GST alpha, mu, pi and theta, stronger staining intensity was observed in normal epithelial cells when compared with tumour cells and statistically it was proved that GST expression was dependent on tissue type (normal, in situ, invasive). Every human cancer that has been analysed reveals multiple genetic alterations resulting in morphological and functional differences from the normal cell. Tumour cells may lose some of their functions (expression of some proteins) in the malignant transformation process. It can be speculated that low level of GST expression in the tumour cells can be a result of this transformation.

In this study, we observed that nuclear staining for GST isoenzymes was patchy and heterogenous in intensity even in an individual tumour section. The enzymatic techniques do not reflect this heterogeneity within tumours. This heterogeneity is an expected finding and it is most likely to be the result of tumour progression. Heterogeneity may influence the responses of tumour cell to chemotherapeutic agents. It may be speculated that tumour cells with a high GST content may be more resistant to cytotoxic and chemotherapeutic agents. Accordingly, in vitro studies have demonstrated that alpha-class GSTs can enhance the conjugation of alkylating agents, such as phenylalanine mustard (melphalan) and chlorambucil, with glutathione (Fairchild et al., 1990; Chin et al., 1990) and mu-class GSTs are capable of inactivating nitrosoureas through denitrosation (Safa et al., 1986). The development of multidrug resistance in the multidrug-resistant MCF-7 human breast cancer cell line was associated with increased GST pi expression (Batist et al., 1986) and increased GST levels also have been found in a variety of other drug-resistant cell lines (Fisher et al., 1981; Wolf et al., 1990). However, the role of GSTs in the development of antineoplastic drug resistance still is not certain. Increased intracellular glutathione(GSH) levels as well as increased activity of GSH-dependent enzymes have been associated with multidrug resistance in some studies, whereas others have failed to find evidence for a role of GSH or its dependent enzymes in multidrug resistance (Fairchild et al.,1990; Moscow et al., 1989; Leyland-Jones et al., 1991). As GSTs isoenzymes are known to have markedly different substrate specificities, the total GST isoenzyme composition

may be important determinants of a tumour cell's ability to detoxify particular antineoplastic compounds. Both the amount and the proportion of different enzymes present in tumours play a role in determining anti-cancer drug resistance. In this study, we did not have the chance to study the possible chance in GST levels before and after treatment in the same patient group but we could not find any difference in GST expression between treated and untreated patients. However, our finding does not rule out the possibility of such a difference which can be determined in a larger study group.

In this study, in normal epithelium there was no difference between the expression of GST isoenzymes. However, in intraductal and invasive tumour tissues expression was dependent on GST isoenzymes. It may be speculated that expression of GST isoenzymes may be actors in negatively or positively selection of tumour cells; and level of GST expression may have effects on prognosis. Accordingly, Gilbert and coworkers demonstrated a negative effect of GST pi expression on prognosis that was independent of ER and PR status, tumour size, nuclear grade, and patient's age.

Increasing age is a significant risk factor for breast cancer. This study indicates that GST alpha expression was dependent on patient's age. No relationship was observed for GST mu, pi and theta. Other researchers reported that no relationship could be found between GSTs expression and age (Howie et al, 1989; Clapper et al, 1991; Gilbert et al, 1993).

In this study, GST theta expression was dependent on the hormone therapy status in breast cancer. No relationship was observed for GST mu, pi and alpha. Soubeyran (1996) observed a significant increase in GST pi expression and a decrease in ER and PR following tamoxifen treatment.

Several reports had shown that GST-pi expression is inversely correlated with ER levels in primary breast cancer. It has been reported that ER and PR negative breast tumours have significantly higher levels of GST-pi mRNA and

protein levels than ER and PR positive tumours (Moscow et al., 1988; Howie et al.,1989; Gilbert et al.,1993). Forrester et al.,(1990) also, reported that there was no association between the level of expression of mu class GST protein with ER status. Other studies suggested that there is no connection between the total GST or GST-pi activity and ER or PR status (Clapper et al.,1991), or is much weaker than previously reported (Shea et al.,1990; Colovai et al., 1992). We did not observe a relationship between GST alpha, mu, pi and theta expressions and oestrogen receptor status.

No research was reported related with microcalcification status and GST expression. In this study, GST alpha and mu expressions were dependent on microcalcification status. No relationship for GST theta and pi was observed.

The ultimate carcinogens of PAH are epoxide-containing metabolites, many of which are substrates for class mu and pi class GST. Nazar-Stewart, et al. (1993) suggested that high GST mu activity was associated with a greater decrease in lung cancer risk among heavy than light cigarette smokers. There is no study on the GST expression and smoking in breast cancer. In this study, no relationship was observed between GSTs expression and smoking status.

In this study, GST alpha and theta expression were dependent on menopausal status. No relationship was observed for GST mu and pi. Similarly, Howie et al (1989) reported that no relationship could be found between GST expression and menopausal status.

In this study, no relationship was found between GST alpha, mu, pi, and theta expressions and tumour stage. Similarly, Howie and coworkers (1989) reported that no relationship could be found between GSTs expressions and tumour stage.

In this study, GST pi expression was dependent on parity. However, GST alpha, mu and theta were independent of parity. There is no study on the GST expression and parity in breast cancer.

The number of breast cancer patients in this analysis is small. Future studies with substantially larger numbers of breast cancer patients will be needed to examine prospectively for the possible relationship between GST expression and prognostic factors.

Western blotting analysis showed that alpha, mu, and pi class of GSTs were expressed in all normal and breast carcinoma tissues which is consistent with Forrester et al, (1990). Class theta GSTs have never been studied in human breast normal and carcinoma by western blotting except in Ph D thesis of Awni AbuHijleh (1999).

In this study, by western blotting analysis, the levels of alpha and mu class GST proteins varied in normal and tumour tissue significantly among individuals. The mu class GST which is usually known as polymorphic GST was expressed in all indivuals in this study. The pi, alpha and theta class enzymes were also prepondrate in all samples. The level of pi class GST protein was found in high concentrations but, GST theta isoenzyme was expressed at low concentrations in all normal and tumour samples.

The overall GST activities were higher in the tumour cytosolic fractions, against both CDNB and EPNP, in all of the 21 patients examined in a previous study (Ergüney P., M.S. thesis, 1999). However, in the western blotting study of the same samples, isoenzyme levels was not higher in tumour tissue in all cases. GST level was higher in tumour tissue when compared with normal tissue in only 20%, 29%, 38% and 10% of the samples for GST alpha, GST mu, GST pi and GST theta respectively. In the remaining cases either normal tissue showed higher level of GST proteins or the protein levels in normal and tumour tissue were similar.

Therefore, GSTs isoenzyme levels are not correlated with their overall enzymatic activity in the normal and tumour cytosolic fractions.

In humans, one member of the mu class gene family glutathione S-transferase M1 (GSTM1) has been shown to be polymorphic and is absent in 35-60% of individuals (Bell, D.A. et al. 1993; Katoh, T. et al. 1995; Groppi, A. et al. 1991). Similarly, glutathione S-transferase T1 (GSTT1), a member of the theta class gene family, is also polymorphic and is absent in 10-65% of human populations (Nelson, H. H. et al. 1995; Elexpuru-Camiruaga, J. et al. 1995; Chenevix-Trench, G. et al. 1995). The phenotypic absence of GSTM1 and GSTT1 activity is due to homozygosity for an inherited deletion of these genes, termed the null genotype (Seidegard, J. et al. 1986; Pemble, S. et al. 1994). Six of the samples (29%) in the previous study (Ergüney P., M.S. thesis, 1999) had no detectable GSTs activity against EPNP. This is most probably associated with the polymorphic expression of the class theta GSTT1-1. However, according to the western blotting analysis of the same cases, GST theta protein was present in all of the samples. Although samples for WB and IHC methods were different, all the normal and tumour samples contain detectable amounts of GST mu and theta isoenzymes in both methods. These findings have not support the polymorphic expressions of GST mu and GST theta protein. Our results may indicate that GST mu and theta protein in this study might be different gene products of GST mu and theta gene class family which would be different member of the mu and theta class gene family GST. Further research will be needed to find genotypic distribution of these proteins in the human breast tissue by in situ hybridisation technique. By this technique, the genes that code for these proteins will be localised in the chromosome and will be compared with the gene locus of GSTM1 and GSTT1.

CHAPTER V

CONCLUSION

In this study, epithelial cells either malignant or non-malignant staining with any of four GST antibodies generally showed a diffuse cytoplasmic staining and patchy nuclear staining in normal breast and tumour tissue. In addition, there is a great heterogeneity in nuclear staining intensity among normal and tumour cells for the GST alpha, mu, pi and theta isoenzymes. Intracellular localisation of the isoenzymes may provide additional information about the functions of the isoenzymes, thus immunohistochemistry is a useful method to study the GST isoenzymes.

For GST alpha, mu, pi and theta, stronger staining intensity was observed in normal epithelial cells when compared with tumour cells and statistically it was proved that GST expression was dependent on tissue type (normal, in situ, invasive). We observed that nuclear staining for GST isoenzymes was patchy and heterogenous in intensity even in an individual tumour section. The enzymatic techniques do not reflect this heterogeneity within tumours. This heterogeneity is an expected finding and it is most likely to be the result of tumour progression. Heterogeneity may influence the responses of tumour cell to chemotherapeutic agents. It may be speculated that tumour cells with a high GST content may be more resistant to cytotoxic and chemotherapeutic agents.

In this study, we did not have the chance to study the possible chance in GST levels before and after treatment in the same patient group but we could not find any difference in GST expression between treated and untreated patients.

In normal epithelium there was no difference between the expression of different GST isoenzymes. However, in intraductal and invasive tumour tissues expression was dependent on GST isoenzymes.

GST alpha expression was dependent on patient's age. GST theta expression was dependent on the hormone therapy status in breast cancer. We did not observe a relationship between GST alpha, mu, pi and theta expressions and oestrogen receptor status. GST alpha and mu expressions were dependent on microcalcification status. No relationship was observed between GSTs expression and smoking status.

GST alpha and theta expression were dependent on menopausal status. No relationship was found between GST alpha, mu, pi, and theta expressions and tumour stage. GST pi expression was dependent on parity.

In the western blotting analysis, the levels of alpha and mu class GST proteins varied in normal and tumour tissue significantly among individuals. The mu class GST which is usually known as polymorphic GST was expressed in all indivuals. The pi, alpha and theta class enzymes were also prepondrate in all inviduals. The level of pi class GST protein was found in high concentrations but, GST theta isoenzyme was expressed at low concentrations in all normal and tumour samples.

The overall GST activities were higher in the tumour cytosolic fractions, against both CDNB and EPNP, in all of the 21 patients examined in a previous study. However, in the western blotting study of the same samples, isoenzyme levels was not higher in tumour tissue in all cases. Therefore, GSTs isoenzyme

levels are not correlated with their overall enzymatic activity in the normal and tumour cytosolic fractions.

In the previous study, 29% of the cytosolic fractions (6 out of 21) had no activity of GSTs against EPNP as a substrate. This is most probably associated with the polymorphic expression of the class theta GSTT1-1 where 35-60% of the human population were reported to be negative conjugators. However, according to the western blotting analysis of the same cases, GST theta protein was present in all of the samples. Although samples for WB and IHC methods were different, all the normal and tumour samples contain detectable amounts of GST mu and theta isoenzymes in both methods. These findings have not support the polymorphic expressions of GST mu and GST theta protein. Our results may indicate that GST mu and theta protein in this study might be different gene products of GST mu and theta gene class family which would be different member of the mu and theta class gene family GST. Further research will be needed to find genotypic distribution of these proteins in the human breast tissue by in situ hybridisation technique. By this technique, the genes that code for these proteins will be localised in the chromosome and will be compared with the gene locus of GSTM1 and GSTT1.

REFERENCES

Abu-Hijleh, A. A., 1999. " Purification, and kinetic and immunologic characterization of theta class glutathione S-transfese GSTT1-1 from normal and cancerous human breast tissue" Ph. D. thesis in Biochemistry, Middle East Technical University-Ankara.

Agrup, G., Falck, B., Rorsman, H., Rosengren, A. M., and Rosengren, E., 1977. "Glutathione dopa in malignant melanoma", *Acta Dermatol.*, Vol. 57, pp.221-226.

Ahmann, D. L., Bisel, H. F., Eagan, R. T., 1974. " Controlled evaluation of Adriamycin in patients with disseminated breast cancer", *Cancer Chemother. Rep.*, Vol.58, pp.877.

Allegra J. C., Barlock, A., Huff, K. K., Lippman, M. E., 1980. " Changes in multiple or sequential oestrogen receptor determinations in breast cancer", *Cancer*, Vol.45, pp.792-794.

Alin, P., Danielson, U. H., and Mannervik, B. 1985. "4-hydroxyalk-2-enals are substrates for glutathione transferases", *FEBS Lett.*, Vol.179, pp.267-270.

Ambrosone, C. B., Freudenheim, J. L., Graham, S., Marshall, J. R., Vena, J. E., Brasure, J. R., Laughlin, R., Nemoto, T., Michalek, A. M., Harrington, A., Ford, T. D., Shields, P. G., 1994. " Cytochrome P4501A1 and Glutathione S-transferase (M1) Genetic Polymorphisms and Postmenopausal Breast Cancer Risk", *Cancer Res.*, Vol.55, pp.3483-3485.

Amenta, P. S., 1986. " Elias-Pauly's Histology and Human Microanatomy", New York, Wiley.

Ames, B. N., 1983. "Dietary carcinogens and anti-carcinogens", *Science*, Vol. 221, pp.1256-1264.

Ames, B. N., Profet, M., and Gold, L. S., " Dietary pesticides",1990. *Proc. Natl. Acad. Sci. U.S.A.*, Vol. 87, pp.7777-7781.

Anttila, S., Hirvoven, A., Vainio, H., Ketterer,B., 1993 "Immunohistochemical localization of glutathione S-transferases in human lung" *Cancer Research* Vol.53 pp.5643-5648.

Arca, P., Hardisson, C., and Suarez, J. E., 1990. " Purification of a glutathione S-transferase that mediates fosfomycin resistance in bacteria", *Antimicrob. Agents Chemother.*, Vol.34, pp. 844-848.

Arey, L. B., 1974. " Human Histology:A textbook in Outline Form", Philadelphia, W. B. Saunders.

Asako, K., Ito, H., and Takahaski, K., 1977. "Monkey glutathione S-aryltransferases", *J. Biochem.*, Vol.82, pp.973-981.

Askelof, P., Guthenberg, C., Jakobson, I., and Mannervik, B., 1975. "Purification and characterization of two glutathione S-aryltransferase activities from rat liver", *Biochem. J.*, Vol. 147, pp.513-522.

Awasthi, Y.C., Singh, S.V., Ahmad,H., Moller, P.C., and Gupta, V., 1988. "Expression of GST isoenzymes in human small cell lung cancer cell lines", *Carcinogenesis.* Vol.9, pp.89-93

Azzopardi, J. G., Chepick, O. F., Hartman, W. H., 1982. " Histologic typing of breast tumours", *Am. J. Clin. Pathol.*, Vol.78, pp.806.

Azzopardi, J. G., 1983. " Benign and malignant proliferative lesions of the breast, a review", *J. Cancer Clin. Oncol.*, Vol.19, pp.1717.

Bankfalvi, A., Navabi, H., Bier, B., Bocker, W., Jasani, B., Schmid, K. W., 1994. "Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry", *Journal of Pathology*, Vol.174, No.3, pp.223-228.

Batist, G., Tulpule, A., Sinha, B.K., Katki, A. G., Myers, C.E., and Cowan, K. H., 1986. "Overexpression of a novel anionic glutathione transferase in multi-drug resistant human breast cancer cells", *J. Biol. Chem.*, Vol. 261, pp. 15544-15549.

Beahrs, O. H., 1991. " Staging of cancer", *Cancer J. Clin.*, Vol.41, pp.121-125.

Beckett, G. J., Boyd, R., Beddows, S. E., and Hayes, J. D., 1988. "Decreased hepatic glutathione S-transferase A, AA, and L concentration produced by prolonged thyroid hormone administration", *Biochem. Pharmacol.*, Vol. 37, pp. 3201-3204.

Beckett, G., and Hayes, J. D., 1993. " Glutathione S-transferases: biomedical applications", *Adv. Clin. Chem.*, Vol.30, pp.279-378.

Bellamy, C. O., Harrison, D. J., 1994. " Evaluation of glutathione S-transferase pi in non-invasive ductal carcinoma of breast" ,*Br. J. Cancer*, Vol.69, pp.183-185.

Bennet C.F:, Yeoman L.C. 1987. "Microinjected glutathione S-transferase Yb subunits translocate to the cell nucleus". *Biochem. J.* Vol.247. pp109-112.

Benson, A. M., Batzinger, R. P, Ou, S-Y. L., Bueding, E., Cha, Y-N, and Talalay, P., 1978. " Elevation of hepatic glutathione S-transferase activities and protection against mutagenic metabolites of benzo[a]pyrene by dietary antioxidants", *Cancer Res.*, Vol.38, pp.4486-4495.

Berhane, K., Hao, X.-Y., Egyhazi, S., Hansson, J., Ringborg, U., and Mannervik, B., 1993. " Contribution of glutathione transferase M3-3 to 1,3-Bis(2-chloroethyl)-1-nitrosourea resistance in a human non-small cell lung cancer cell line", *Cancer Res.*, Vol.53, pp.4257-4261.

Bezwoda, W. R., Esser, J. D., Dansey, R., 1991. " The value of estrogen and progesterone receptor determinations in advanced breast cancer: Estrogen receptor level but not progesterone receptor level coorelates with response to tamoxifen", *Cancer*, Vol.68, pp.867.

Black, S. M., Beggs, J. D., Hayes, J. D., Bartoszek, A., Muramatsu, M., Sakai, M., and Wolf, C. R., 1990. " Expression of human glutathione S-transferases in S. *cerevisiae* confers resistance to the anticancer drugs adriamycin and chlorambucil", *Biochem. J.*, Vol.268, pp.309-315.

Bloom, N. D., and Fishman. J. H., 1983. " Tamoxifen treatment failures in hormonally responsive breast cancers. Correlation with steroid receptors", *Cancer*, Vol.51, pp.1190.

Blum, H., Beier, H., and Gross, H. J., 1987. "Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels", *Electrophoresis*, Vol.18, pp.93-99

Board, P. G., Coggan, M., Wilce, M. C. J., and Parker, M. W., 1995. " Evidence for an essential serine residue in the active site of the theta class glutathione transferases", *Biochem. J.*, Vol.311, pp.247-250.

Bogaards, J. J. P., van Ommen, B., and van Bladeren , P. J., 1993. " Interindividual differences in the in vitro conjugation of methylene chloride with glutathione by cytosolic glutathione S-transferase in 22 human liver samples", *Biochem. Pharmacol.*, Vol.45, pp.2166-2169.

Board, P. G., 1981. " Biochemical genetics of glutathione S-transferase in man", *Am. J. Hum. Genet.*, Vol.33, pp.36-43.

Board, P. G. and Webb, G. C., 1987. " Isolation of a cDNA clone and localization of human glutathione S-transferase 2 genes to chromosome band 6p12", *Proc. Natl. Acad. Sci. U.S.A.*, Vol.84, pp.2377-2381.

Board, P. G., Webb, G. C., and Coggan, M. C., 1989. " Isolation of a cDNA clone and localization of the human glutathione S-transferase 3 genes to chromosome bands 11q13 and 12q13-14", *Ann. Hum. Genet.*, Vol.53, pp.205-213.

Board, P. G., Coggan, M., and Woodcock, D. M., 1992. " The human pi class glutathione transferase sequence at 12q13-q14 is a reverse-transcribed pseudogene", *Genomics*, Vol.14, pp.470-473.

Buetler, T. M. and Eaton, D. L., 1992. " Glutathione S-transferases: amino acid sequence comparison, classification and phylogenetic relationship", *Environ. Carcinogen. Ecotoxicol Revs.*, Vol.C10, pp.181-203.

Buetler, T. M., and Eaton, D. L., 1992. " Complementary DNA cloning, messenger RNA expression, and induction of α-class glutathione S-transferases in mouse tissues", *Cancer Res.*, Vol.52, pp.314-318.

Buller, A. L., Clapper, M. L., Tew, K. D., 1987. "Glutathione S-transferases in nitrogen mustard-resistant and sensitive cell lines", *Mol. Pharmacol.*, Vol.31, pp.575-578.

Cairns, J., Wright, C., Cattan, A. R., *et al.* 1992," Immunohistochemical demonstration of glutathione S-transferases in primary human breast carcinomas" *J. of Pathology* Vol.166, pp.19-25.

Campbell, J. A. H., Corrigal, A. V. , Guy, A., Kirsch R. E., 1991. "Immunohistologic localization of alpha, mu, and pi class glutathione S-transferases in human tissues" *Cancer*, Vol.67, pp.1608-1613.

Carmichael, J., Forrester, L. M., Lewis, A. D., Hayes, J.D., Hayes, P. C., and Wolf, C. D., 1988. "Glutathione S-transferases isoenzymes and glutathione peroxidase activity in normal and tumor samples from human lung" *Carcinogenesis*, Vol.9 No.9, pp. 1617-1621.

Carter, C. L., Allen, C., and Henson, D. E., 1989. " Relation of tumour size, lymph node status, and survival in 24730 breast cancer cases", *Cancer*, Vol.63, pp.181-187.

Cattoretti, G., Pileri, S., Parravicini, C., Becker, M. H. G., Poggi, S., Bifulco, C., Key, G., Damato, L., Sabattini, E., Feudale, E., Reynolds, F., Gerdes, J., Rilke, F., 1993. "Antigen unmasking on formalin-fixed, paraffin-embedded tissue-sections", *Journal of pathology*, Vol.171, No.2, pp.83-98.

Chambers, T. C., McAvoy, E. M., Jacobs, J. W., Eilon, G., 1990. " Protein kinase C phosphorylates P-glycoprotein in multidrug resistant human KB carcinoma cells", *J. Biol. Chem.*, Vol.265, pp.7679-7686.

Chang, L. H., Chuang, L. F., Tasi, C. P., Tu, C. P. D., and Tam, M. F., 1990. "Characterization of Glutathione S-transferases from day-old chick livers", *Biochemistry*, Vol. 29, pp.744-750.

Chasseaud, L. F., 1979." The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents", *Adv. Cancer. Res.*, Vol.29, pp.175-274.

Chenevix-Trench, G., Young, J., Coggan, M., and Board, P., 1995. " Glutathione S-transferase M1 and T1 polymorphisms: susceptibility to colon cancer and age of onset", *Carcinogenesis*, Vol.16, pp.1655-1657.

Chin, K., Tanaka, S., Darlington, G., Pastan, I., Gottesman, M. M., 1990. " Heat shock and arsenite increase expression of the multidrug resistance (MDR) gene in human renal carcinoma cells", *J. Biol. Chem.*, Vol.265, pp.221-226.

Clapper M. L., Hoffman, S. J., Carp, N., Watts, P., Seestaller, L. M., Weese, J. L., Tew, K. D., 1991. " Contribution of patient history to the glutathione S-transferase activity of human lung, breast and colon tissue", *Carcinogenesis*, Vol.12, No.10, pp.1957-1961.

Clarck, A. G., Smith, J. N., and Speir, T.W., 1973. "Cross specificity in some vertebrate and insect glutathione transferases with methylparathion (dimethyl p-nitro-phenyl phosphorothionate), 1-crotonyl-N-acetylcysteamine as substrates", *Biochem. J.*, Vol.135, pp.385-392.

Clarck, A.G., and Dauterman, W.C., 1982. "The characterization by affinity chromatography of glutathioneS-transferases from different strains of housefly", *Pestic. Biochem. Physiol.*, Vol.17, pp.307-314.

Colovai, A. I., Stoica, A., Cinca, S. A., Marin, M., Gruia, M., Condrea, I., Petrescu, D., Dutu, R., 1992. " Glutathione S-transferase activity in human breast tumours", *Neoplasma*, Vol.39, No.4, pp.249-254.

Cotran, Kumar, Robbins, 1994. " Robbins Pathologic Basis of Disease", Saunders Company.

Corrigall, A.V., and Kirsch, R.E., 1988 "Glutathione S-transferase distribution and concentration in human organs" *Biochem. Inter.* Vol.16 No.3, pp. 443-448.

Cowan, K. H., Batist, Tulpule, A., Sinha, B. K., Myers, C. E., 1986. "Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats", *Proc. Natl. Acad. Sci. USA*, Vol.83, pp.9328-9332.

Curran, R. C., Gregory, J., 1977. "The unmasking of antigens in paraffin sections of tissue by trypsin". *Experientia* Vol.33, pp.1400-1401.

Daly, A. K., Thomas, D. J., Cooper, J., Pearson, W. R., Neal, D. E., and Idle, J. R., 1993. " Homozygous deletion of gene for glutathione S-transferase M1 in bladder cancer", *Br. Med. J.*, Vol.307, pp.481-482.

Danger, D. P., Baldwin, W. S., and LeBlanc, G. A., 1992. " Photoaffinity labelling of steroid-hormone-binding glutathione S-transferases with [^{3}H]methyltrienolone. Inhibition of steroid-binding activity by the anticarcinogen indole-3-carbinol", *Biochem. J.*, Vol. 288, pp.361-367.

Danielson, U. H., Esterbauer, H., and Mannervik, B., 1987. "Structure-activity relationships of 4-hydroxyalkenals in the conjugation catalysed by mammalian glutathione transferases" *Biochem. J.*, Vol.247, pp.707-713.

Davies, M. H., Elias, E., Acharya, S., Cotton, W., Faulder, G. C., Fryer, A. A., and Strange, R. C., 1993. " GSTM1 null polymorphism at the glutathione S-transferase M1 locus: phenotype and genotype studies in patients with primary biliary cirrhois", *Gut*, Vol.34, pp.549-553.

De Waziers, I., Cugnenc, P.H., Berger, A., 1991 " Drug-metabolizing enzyme expression in human normal, peritumoral and tumoral colorectal tissue samples" *Carcinogenesis*, Vol. 12, No.5 pp.905-909.

Deffie, A. M., Alam, T., Seneviratne, C., Beenken, S. W., Batra, J. K, Shea, T. C., Henner, W. D., and Goldenberg, G. J., 1988. "Multifunctional resistance to adriamycin: relationship of DNA repair, glutathione transferase activity, drug efflux, and P-glycoprotein in cloned cell lines of adriamycin sensitive and resistant p388 leukemia" , *Cancer Res.*, Vol.48, pp.2595-2602.

Del Boccio, G., Di Ilio, C., Alin, P., Jornvall, H., and Mannervik, B., 1987. " Identification of a novel glutathione transferase in human skin homologous with class alpha glutathione transferase 2-2 in the rat", *Biochem. J.*, Vol.244, pp.21-25.

Di Ilio, C., Polidoro, G., Arduini, A., and Federici, G., 1982. "Glutathione S-transferase activity from guinea pig brain-A comparison with hepatic multiple forms", *Gen. Pharmacol.*, Vol.13, pp.485-490.

Di Ilio, C., Sacchetta, P., Del Boccio, G., La Rovere, G., and Federici, G. 1985 "Glutathione peroxidase, glutathione S-transferase and glutathione reductase activities in normal and neoplastic human breast tissue" *Cancer Lett.*, Vol.29 pp.37-42.

Di Ilio, C., Del Boccio, G., Aceto, A., and Federici, G., 1987. " Alteration of glutathione transferase isoenzyme concentrations in human renal carcinomas", *Carcinogenesis* Vol.8, pp.861-864.

Di Ilio, C., Del Boccio, G., Aceto, A., Casaccia, R., 1988 "Elevation of Glutathione transferase activity in humon lung tumor". *Carcinogenesis,* Vol. 9, No.2 pp.335-340.

Di Ilio, C., Aceto, A., Bucciarelli, T., Dragani, B., Angelucci, S., Miranda, M., Poma, A., Amicarelli, F., Barra, D., and Federici, G., 1992. " Glutathione transferase isoenzymes from Bufo bufo embryos at an early developmental stage", *Biochem. J.,* Vol.283, pp.217-222.

Dixon, J. M., Anderson, T. J., Page, D. L., 1983. " Infiltrating lobular carcinoma of the breast. An evaluation of the incidence and consequences of bilateral disease", *Br. J. Surg.,* Vol.70, pp.513.

Dominey, R. J., Nimmo, I. A., Cronshaw, A. D., and Hayes, J. D., 1991. " The major glutathione s-transferase in Salmonid fish livers is homologous to the mammalian pi-class GST", *Comp. Biochem. Physiol.,* Vol.B100, pp.93-98.

Donegan, W. L., and Perez-Mesa, C. M., 1972. " Lobular carcinoma: An indicator for elective biopsy of the second breast", *Ann. Surg.,* Vol.177, pp.178-187.

Donegan, W. L., 1992. " Prognostic Factors: Stage and receptor status in breast carcer", *Cancer,* Vol.70, pp.1755-1764.

Dorion-Bonnet, F., Quenel, N., Coindre, J. M., Mauriac, L., Bonichon, F., Duran, M., Wafflart, J., Moscow, J. A., Cowan, K. H., Gualde, N., 1993. " Expression of the GST pi gene and response to tamoxifen therapy in locally advanced breast carcinomas", *Ann. NY. Acad. Sci.,* Vol.698, pp.182-185.

Dusre, L., Mimnaugh, E. G., Myers, C. E., Sinha, B. K., 1989. " Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumour cells", *Cancer Res.*, Vol.49, pp.511-515.

Early Breast Cancer Trialists' Collaborative Group, 1992. " Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy", *Lancet*, Vol.339, pp.1-15, 71-85.

Eimoto, H., Tsutsumi, M., Nakajima, A., Yamamoto, K., 1988 " Expression of the glutathione S-transferase placental form in human lung carcinomas" *Carcinogenesis* Vol.9 No.12 pp.2325-2327.

Elexpuru-Camiruaga J., Buxton N., Kandula V., 1995. "Susceptibility to astrocytoma and meningioma:influence of allelism at GSTT1 and GSTM1 and cytochrome P450".*Cancer Res.*Vol.55 pp.4237-4239.

Ellis, I. O., Galea, M., Broughton, N., 1992. " Prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up", *Histopathology*, Vol.20, pp.479.

El Mouelhi, M., Diolkar, M. S.,Elias E.G., Guengerich E.G. 1987. "Hepatic drug-metabolizing enzymes in primary and secondary tumours of human liver". Cancer Research. Vol.47, pp 460-466.

Elston, C. W. "Grading of invasive carcinoma of the breast". In: Page, D. L., Anderson, T. J. eds. *Diagnostic Histopathology of the Breast*. Edinburgh: Churchill Livingstone, 1987; 300-311.

Ergüney, P.,1999. "GST theta in Breast Tissue" M. S. thesis, Middle East Technical University-Ankara

Evans, C. G., Bodell, W. J., Tokuda, K., Doane-Setzer, P., Smith, M. T., 1987. " Glutathione and related enzymes in rat brain tumor cell resistance to 1,3-Bis(2-chloroethyl)-1-nitrosourea and nitrogen mustard", *Cancer Res.*, Vol.47, pp.2525-2530.

Evers, P., Uylings, H.B.M., 1994. "Microwave-stimulated antigen retrieval is pH and temperature-dependent", *Journal of Histochemistry and Cytochemistry*, Vol.42, No.12, pp.1555-1563.

Farber, E., 1990. " Clonal adaptation during carcinogenesis", *Biochem. Pharmacol.*, Vol.39, pp.1837-1846.

Faulder, C.G., Hirrel, P. A., Hume, R., and Strange, R. C., 1987. " Studies on the development of basic, neutral and acidic isoenzymes of glutathione S-transferase in human liver, adrenal, kidney and spleen", *Biochem. J.*, Vol.241, pp. 221-228.

Fechner, R. E., and Mills, S. E., 1990. " Breast Pathology", Chicago, ASCP Press.

Fairchild, C. R., Moscow, J. A., O'Brien, E. E., Cowan, K. H., 1990. " Multidrug resistance in cells transfected with human genes encoding a variant P-glycoprotein and glutathione S-transferase pi", *Mol. Pharmacol.*, Vol.37, pp.801-809.

Fentiman, I. S., Lavelle, M. A., Caplan, D., 1986. " The significance of supraclavicular fossa node recurrence after radical mastectomy", *Cancer*, Vol.57, pp.908.

Fisher, E. R., Gregorio, R. M., Fisher, B., 1975. " The pathology of invasive breast cancer: a syllabus derived from findings of the National Surgical Adjuvant Breast Project (Protoco No. 4)". *Cancer,* Vol.36, pp.1.l

Fisher, E. R., Palekar, A. S., Redmond, E., 1980. " Pathologic findings from the national surgical adjuvant breast project (No.4) VI Invasive Papillary Carcinoma", *Am. J. Clin. Pathol.,* Vol.73, pp.313.

Fisher, E. R., Kent, O., McGuire, W. L., 1981. " Correlation of primary breast cancer histopathology and oestrogen receptor content", *Breast Cancer Res. Treat.,*Vol.1, pp.37-41.

Fisher, E. R., Costantino, J., Fisher, B., 1992b. " Pathologic findings from the National Surgical Adjuvant Breast Project (Protocol 4)", *Cancer,* Vol.71 (Suppl), pp.2141-2150.

Fisher, B., 1992. " Experimental and clinical justification for the use of tamoxifen in a breast cancer prevention trial", *Proc. Am. Assoc. Cancer Res.,* Vol.33, pp.567.

Fisher, E. R., Anderson, S. Redmond, C., 1993. " Pathologic findings from the National Surgical Adjuvant Breast Project Protocol-B-06. 10-year pathologic and clinical prognostic discriminants", *Cancer,* Vol.76, pp.2507.

Forrester, L.M., Hayes, J.D., Millis, R., Barnes, D., Harris, A.L., 1990 "Expression of glutathione S-transferases and cytochrome P450 in normal and tumor breast tissue" *Carcinogenesis* Vol.11 No.12 pp. 2163-2170.

Foureman and Bend, 1984. " The hepatic glutathione transferases of the male litle skate, Raja-Erinacea", *Chem. Biol. Interact.,* Vol.49, pp. 89-103.

Fournier, D., Bride, J. M., Poirie, M., Berge, J.-B., and Plapp, Jr., F. W., 1992. " Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides", *J. Biol. Chem.*, Vol.267, pp.1840-1845.

Fryer A.A., Zhao L., 1993. "The glutathione S-transferases; polymerase reaction studies on the frequency of the GSTM10 genotype in patients withadenoma". *Carcinogenesis* Vol.14.pp.563-566.

Fujita, S., Kitagawa, H., Ishizawa, H., Suzuki, T., and Kitani, K., 1985. "Age associated alterations in hepatic glutathioneS-transferase activities". *Biochem. Pharmacol.*, Vol.34, pp.3891-3894.

Fuqua, S. A., 1992. " Where is the lesion in hormone-independent breast cancer?" (Editorial), *J. Natl. Cancer Inst.*, Vol.84, pp.554.

Giaccia, A. J., Lewis, A. D., Denko, N. C., Cholon, A., Evans, J. W., Waldren, C. A., Stamato, T. D., 1991. " The hypersensitivity of the Chinese hamster ovary variant BL-10 to bleomycin killing is due to lack of glutathione S-transferase-alpha activity", *Cancer Res.*, Vol.51, pp.4463-4469.

Gilbert, L., Elwood, L. J., Merino, M., Masood, S., Barnes, R., Steinberg, S. M., Lazarous, D. F., Pierce, L., d'Angelo, T., Moscow, J. A., Townsend, A. J., and Cowan, K. H., 1993. " A pilot study of pi-class glutathione S-transferase expression in breast cancer: correlation with estrogen receptor expression and prognosis in node-negative breast cancer", *J. Clin. Oncol.*, Vol.11, pp.49-58.

Gold, R. H., Main, G., Zippin, C., 1972. " Infiltration of mammary carcinoma as an indicator of axillary metastasis: A preliminary report", *Cancer*, Vol.29, pp.35.

Graves, R. J., Coutts, C., Eyton-Jones, H., and Green, T., 1994. " Relationship between hepatic DNA damage and methylene chloride-induced hepatocarcinogenicity in B6C3F1 mice", *Carcinogenesis*, Vol.15, pp.991-996.

Graves, R. J., Coutts, C., and Green, T., 1995. " Methylene chloride-induced liver damage; an interspecies comparison", *Carcinogenesis*, Vol.16, pp.1919-1926.

Green, J. A., Robertson, L. J., and Clark, A. H., 1993. " Glutathione S-transferase expression in benign and malignant ovarian tumors", *Br. J. Cancer,* Vol.68, pp.235-239.

Greenspan, E. M., 1966. " Combination cytotoxic chemotherapy in advanced disseminated breast carcinoma", *J. Mt. Sinai Hosp.*, Vol.33, pp.1.

Grignon, D. J., Abdel-Malak, M., Mertens, W. C., Sakr, W. A., and Shepherd, R. R., 1994. " Glutathione S-transferase expression in renal cell carcinoma: a new marker of differentiation", *Mod. Pathol.*, Vol.7, pp.186-189.

Grove, G., Zarlengo, R. P., Timmerman, K. P. Li, N.-q., Tam, M. F., and Tu, C.-P. D., 1988. " Characterization and heterospecific expression of cDNA clones of genes in the maize GSH S-transferase multigene family", *Nucleic Acid Res.*, Vol.16, pp.425-438.

Guesdon JL, Ternynck T., Avrameas S.,1979. "The use of avidin-biotin interaction in immunoenzymatic techniques." *J. of Histochem. Cytochem*. Vol.27, p.1131.

Hall, A. G., Autzen, P., Cattan, A. R., Malcolm, A. J., Cole, M., Kernahan, J., and Reid, M. M., 1994. " Expression of mu class glutathione S-transferase correlates with event-free survival in childhood acute lymphoblastic leukemia", *Cancer Res.*, Vol.54, pp.5251-5254.

Hall, P.A., Lane, D.P., 1994. " P53 in tumor pathology-Can we trust immunohistochemistry-revisited", *Journal of pathology*, Vol.172, No.1, pp.1-4.

Hallier, E., Langhof, T., Dannappel, D., Leutbecher, M., Schroder, K., Goergens, H. W., Muller, A., and Bolt, H. M., 1993. " Polymorphism of glutathione conjugation of methyl bromide, ethylene oxide and dichloromethane in human blood: influence on the induction of sister chromatid exchanges (SCE) in lymphocytes", *Arch. Toxicol.*, Vol.67, pp.173-178.

Halliwell, B. and Gutteridge, J. M. C., " Free Radicals in Biology and Medicine", 1985. *Clarendon Press, Oxford.*

Hamada, S.-I., Kamada, M., Furumoto, H., Hirao, T., and Aono, T., 1994. " Expression of glutathione S-transferase-pi in human ovarian cancer as an indicator of resistance to chemotherapy", *Gynecol. Oncol.*, Vol.52, pp.313-319.

Hamilton, T. C., Ozols, R. F.., Dabrow, M. B., 1990. *Oncology*, Vol.4, pp.101.

Harrison, D. J., Kharbanda, R., Bishop, D., McLellan, L. I., and Hayes, J. D., 1989. " Glutathione S-transferase isoenzymes in human renal carcinoma demonstrated by immunohistochemistry", *Carcinogenesis*, Vol.10, pp.1257-1260.

Harada S., Misawa S., 1992. "Detection of GST1 gene deletion by the polymerase chain reaction and correlation with stomach cancer in Japanese". *Hum. Genetics* Vol.90.

Hawkins, R. A., Tesdale, A. L., Anderson, E. D. C., 1990. " Does the oestrogen receptor concentration of a breast cancer change during systemic therapy?", *Br. J. Cancer*, Vol.61, pp.877.

Hayes, J. D. and Mantle, T. J., 1986. " Inhibition of hepatic and extrahepatic glutathione S-transferases by primary and secondary bile acids", *Biochem. J.*, Vol.233, pp.407-415.

Hayes, J. D. and Mantle, T. J., 1986b. "Use of immunoblot techniques to discriminate between the glutathione S-transferase Yf, Yk, Yg, Yn/Yb and Yc subunits and to study their distribution in extra-hepatic tissues", *Biochem. J.*, Vol.223, pp.779-778.

Hayes, P.C., Harrison D.J., Bouchier, I.A.D., McLellan, L.I., 1989. "Cytosolic and microsomal glutathione S-transferase isoenzymes in normal human liver and intestinal epithelium" *Gut*, Vol.30, pp.854-859.

Hayes, J. D. and Wolf, C. R., 1990. " Molecular mechanisms of drug resistance", *Biochem. J.*, Vol. 272, pp.281-295.

Hayes, P.C., Harrison D.J., 1990 "Immunohistochemical analysis of pancreas and gastrointestinal tract in man". in *Glutathione S-transferases and Drug Resistance.*, Taylor and Francis, eds., pp.441-450.

Hayes, P.C., Bouchier, I. A. D., Beckett, G.J., 1991. "Glutathione S-transferase in humans in health and disease" *Gut*, Vol.32, pp.813-818.

Hayes, J. D., Nguyen, T., Judah, D. J., Petersson, D. G., and Neal, G. E., 1994. " Cloning of cDNAs from fetal rat liver encoding glutathione S-transferase Yc polypeptides. The Yc_2 subunit is expressed in adult rat liver resistant to the hepatocarcinogen aflatoxin B_1", *J. Biol. Chem.*, Vol.269, pp.20707-20717.

Hayes, D. F., Wolfe, I. C. H., 1993. " Atlas of Breast Cancer",

Heagerty, a. H. M., Fitzgerald, D., Smith, A., Bowers, B., Jones, P., Fryer, A. A., Zhao, L., Alldersea, J., and Strange, R. C., 1994. " Glutathione S-transferase GSTM1 phenotypes and protection against cutaneous tumors", *Lancet,* Vol.343, pp.266-268.

Henderson J.C., 1990. "Risk factors for breast cancer development".*Cancer* Vol.71.pp.2127-2140.

Henry J. A., Nicholson, S., Hennesy, C., Lennard, T. W. J., May, F. E. B., Westley, B. R., 1989. " Expression of the oestrogen regulated pNR-2 mRNA in human breast cancer: relation to oestrogen receptor m RNA levels and response to tamoxifen therapy", *Br. J. Cancer,* Vol.61, pp.32-38.

Henry, J. A., Piggott, N. H., Mallick, U. K., Nicholson, S., Farndon, J. R., Westley, B. R., May, F. E. B., 1991. " pNR-2/pS2 immunohistochemical staining in breast cancer: correlation with prognostic factors and endocrine response", *Br. J. Cancer,* Vol.63, pp.615-622.

Hiley, C., Fryer, A., Bell, J., Hume, R., Strange, R.C., 1988. "The human glutathione S-transferases" *Biochem J.*, Vol.254, pp. 255-259.

Hiratsuka, A., Sebata, N., Kawashima, K., Okuda, H., Ogura, K., Watabe, T.,Satoh, K., Hatayama, I., Tsuchida, S., Ishikawa, T., and Sato, K., 1990. " A new class of rat glutathione S-transferase Yrs-Yrs inactivating reactive sulfate esters as metabolites of carcinogenic arylmethanols" *J. Biol. Chem.*, Vol.265, pp.11973-11981.

Hirvonen,A., Anttila, S., 1993 " The GSTM1 null genotype as a potential risk modifier for squamous cell carcinoma of the lung" *Carcinogenesis* Vol.14 No.7 pp.1479-1481.

Holmes, F. A., Walters, R. S., Theriault, R. L., 1991. " Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer", *J. Nat. Cancer Insti.*, Vol.83, pp.1797-1805.

Homma H., Listowsky I. "Identification of Yb-glutathione S-transferase as a major rat liver protein labelled with dexamethasone 21-methanesulfonate." 1985. *Proc. Natl. Acad. Sci. USA*. Vol.82. pp 7165-7169.

Howie, A.F., Miller, W.R., Hawkins, R.A., Hutchinson, A.R., Beckett G.J., 1989. "Expression of GSTB1, B2, mu, pi, in breast cancers and their relationship to oestrogen receptor status" *Br. J. Cancer*, Vol.60 pp.834-837.

Howie, A.F., Bell, D., Hayes, P.C., 1990 " Glutathione S-transferase isoenzymes in human bronchoalveolar lavage:a possible early marker for the detection of lung cancer" *Carcinogenesis* Vol. 11 No. 2 pp.295-300.

Howie, A.F., Forrester, L.M., Glancey,M.J., Schlager, J.J., Powis, G., Beckett, G.J., Hayes, J.D., and Wolf, C.R., 1990. "Glutathione S-transferase and glutathione peroxidase expression in normal and tumor human tissues" *Carcinogenesis* Vol.11 No.3 pp.451-458.

Huang, S., Minassian, H., More, J.D., 1976. "Application of immunofluorescent staining in paraffin sections improved by trypsin digestion", *Laboratory Investigation*, Vol.35, pp.383-391.

Hugles, L. E., Mansel, R. E., and Webster, D. J., 1988. " Benign Disorders and Diseases of the Breast: Concepts and Clinical Management", Philadelphia, Bailliere-Tindall.

Hull III, D. F., Clark, G. M., Osborne, K., Chamness, C., Knight III, W. A., McGuire, W. L., 1983. " Multiple oestrogen receptor assays in human breast cancer", *Cancer Res.*, Vol.43, pp.413-416.

Hurlimann, J., Gebhard, S., Gomez, F., 1993. " Oestrogen receptor, progesterone receptor, pS2, ERD5, HSP27 and cathepsin D in invasive ductal breast carcinomas", *Histopathology*, Vol.23, pp.239-248.

Huskey, S.-E. W., Huskey, W. P., and Lu, A. Y. H., 1991. " Contributions of thiolate "desolvation" to catalysis by glutathione-S-transferase isoenzymes 1-1 and 2-2: evidence from kinetic solvent isotope effects", *J. Am. Chem. Soc.*, Vol.113, pp.2283-2290.

Hussey, and Hayes, 1992 "Nomenclature for human glutathione transferases" *Biochem J.* Vol.282 pp.305-308.

Hussey, A. J., and Hayes, J. D., 1992. " Characterization of a human class theta glutathione S-transferase with activity towards 1-menaphthyl sulphate", *Biochem. J.*, Vol.286, pp.929-935.

Igarashi, T., Tomihari, N., Ohmori, S., Ueno, K., Kitagawa, H., and Satoh, T., 1986. "Comparison of glutathione S-transferases in mouse, guinea pig, rabbit and hamster liver cytosol to those in rat liver", *Biochem. Internat.*, Vol.13, No.4, pp.641-648.

Igarashi, T., Irokawa, N., Ono, S., Ueno, K., and Kitagawa, H., 1987. "Difference in the effects of phenobarbital and 3-methylcholanthrene on subunit composition of hepatic glutathione S-transferase in male and female rats", *Xenobiotica*, Vol.17, pp. 127-137.

Igwe, O. J.,1986. "Biologically active intermediates generated by reduced glutathione conjugation pathway: Toxicological implications", *Biochem. Pharmacol.*, Vol.35, pp.2987-2994.

Ingle, J. N., Ahmann, D. L., Green, S. J., 1981. " Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer", *N. Engl. J. Med.*, Vol.304, pp.16.

Ingle, J. N., Krook, J. E., Green, S. J., 1986. " Randomized trial of bilateral oophorectomy versus tamoxifen in premenopausal women with metastatic breast cancer", *J. Clin. Oncol.*, Vol.4, pp.178.

Irwin, C., O'Brien, J. K., Chu, P., Townsend-Parchman, J. K., O'hara, P., and Hunter, F.E., 1980. "Glutathione peroxidase-II of guinea pig liver cytosol-Relationship to glutathione S-transferases", *Arch. Biochem. Biophys.*, Vol. 205, pp.122-131.

Ishigaki, S., Abramovitz, M., and Listowsky, I., 1989. " Glutathione S-transferases are major cytosolic thyroid hormone binding proteins", *Arch. Biochem. Biophys.*, Vol.273, pp.265-272.

Ishikawa, T., Esterbauer, H., and Sies, H., 1986. "Role of cardiac glutathione transferase and of the glutathione S-conjugate export system in biotransformation of 4-hydroxynonenal in the heart", *J. Biol. Chem.*, Vol.261, pp.1576-1581.

Iyer, R. S., Coles, B. F., Raney, K. D., Thier, R., Guengerich, F. P., and Harris, T. M., 1994. " DNA adduction by the potent carcinogen aflatoxin B_1: mechanistic studies", *J. Am. Chem. Soc.*, Vol.116, pp.1603-1609.

İşcan, M., Çoban, T., Bülbül, D., Eke, B. C., Aygörmez, A., Berberoğlu, U., 1998. "The xenobiotic metabolizing and antioxidant enzymes in normal and neoplastic human breast tissue", *Eur. J. Metab. Pharmacokinetics.*

Jakoby, W. B., 1978. " The glutathione S-transferases: a group of multifunctional detoxification proteins", *Adv. Enzymol.*, Vol.46, pp.383-414.

Ji, X., Zhang, P., Armstrong, R. N., and Gilliland, G. L., 1992. " The three-dimensional structure of a glutathione-S-transferase from the mu gene class. Structural analysis of the binary complex of isoenzyme 3-3 and glutathione at 2.2 A resolution", *Biochemistry*, Vol.31 pp.10169-10184.

Joensuu, H., Toikkanen, S., Klemi, P. J., 1990. " DNA index and S-phase fraction and their combinations as prognostic factors in operable ductal breast carcinoma", *Cancer*, Vol.66, pp.331-340.

Juronen, E., Tasa, G., Mart, U., Pooga, M., and Mikelsaar, A-V., 1996. " Production and characterization of monoclonal antibodies against class theta glutathione S-transferase T1-1", *Hybridoma*, Vol.15, No.1, pp.77-82.

Kamby, C., Rasmussen, B. B., and Krinstensen, B., 1989. " Oestrogen receptor status of primary breast carcinomas and their metastases. Relation to pattern of spread and survival after recurrence", *Br. J. Cancer*, Vol.60, pp.252.

Kamisaka, K., Habig, W. H., Ketley, J.N., Arias, M., and Jakoby, W. B., 1975. "Multiple forms of human glutathione S-transferase and their affinity for bilirubin", *Eur. J. Biochem.*, Vol.60, pp.153-161.

Kaplowitz N., Percy-Robb IW, Javitt NB. 1973 "Role of hepatic anion-binding protein in bromosulphthalein conjugation" *J. Exp. Med.* Vol.138 pp.483-487.

Karcioglu, Z. A., and Someren, A., 1985. " Practical Surgical Pathology", Lexington, MA, D. C. Heath & Co, pp.553-635.

Kardinal, C. G., Perry, M. C., Korzun, A. H., 1986. " Lack of differential response of oestrogen receptor-positive vs. ER negative breast cancer to Cytoxan and Adriamycin and 5-fluorouracil chemotherapy (Abstract)", *Proc. Am. Soc. Clin. Oncol.*, Vol.5, pp.74.

Katoh T., Inatomi H., Nagaoka A., 1995. "Cytochorome P450IAI gene polymorphism and homozygous deletion of the glutathione –S-transferase M1 gene in urothelial cancer patients".*Carcinogenesis* Vol.16.pp.655-657.

Katzenellenbogen, B. S., Miller, M. A., Mullick, A., Sheen, Y. Y., 1985. " Antioestrogen action in breast cancer cells: modulation of proliferation and protein synthesis, and interaction with oestrogen receptors and additional antioestrogen binding sites", *Breast Cancer Res. Treat.*, Vol.5, pp.231-243.

Katzellenbogen, B. S., 1991. " Antiestrogen resistance: Mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy (Editorial)", *J. Natl. Cancer Inst.*, Vol.83, pp.1434.

Kelley, M. K., Engqvist-Goldstein, A., Montali, J. A., Wheatley, J. B., Schmidt, D. E., Kauvar, L. M., Jr. 1994. " Variability of glutathione S-transferase isoenzyme patterns in matched normal and cancer human breast tissue", *Biochem. J.*, Vol.304, pp.843-848.

Keizer, H. G., Van Rijn, J., Pinedo, H. M., Joenje, H., 1988. " Effect of endogenous glutathione, superoxide dismutases, catalase, and glutathione peroxidase on adriamycin tolerance of chinese hamster ovary cells", *Cancer Res.*, Vol.48, pp.4493-4497.

Kera, Y., Sippel, H.W., Penttila, K.E., and Lindros, K.O., 1987. "Acinar distribution of glutathione dependent detoxifying enzymes. Low glutathione peroxidase activity in perivenous hepatocytes", *Biochem. Pharmacol.*, Vol.36, pp.2003-2006.

Ketterer, B., Ross-Mansell, P., and Whitehead, J. K., 1967. " The isolation of carcinogen-binding protein from livers of rats given 4-dimethylaminoazobenzene", *Biochem. J.*, Vol.103, pp. 316-324.

Ketterer, B., Harris, J.M., Talaska, G., Meyer, D.J., Pemble, S.E., Taylor, J.B., Lang, N.P., and Kadlubar, F.F.,1992. "The human glutathione S-transferase supergene family, its polymorphism, and its effects on susceptibility to lung cancer" *Environmental Health Perspectives* Vol.98, pp.87-94

Kodate, C., Fukushi, A., Narita, T., Kuda, H., Soma, Y., and Sato, K., 1986. "Human placental form of GST as new immunohistochemical marker for human colonic carcinoma" *Jpn. J. Cancer Res.* Vol.77, pp.226-229.

Korzekwa, K. R., and Jones, J. P., "Predicting the cytochrome P450 mediated metabolism of xenobiotics", 1993. *Pharmacogenetics,* Vol. 3 pp.1-18.

Koskelo, K., 1983. " Isoelectric focusing of glutathione S-transferases: comparison of the acidic transferases from human liver, kidney, lung, spleen, and placenta", *Scand. J. Clin. Lab. Invest.*, Vol.43, pp.133-139.

Kramer, R. A., Zakher, J., Kim, G., 1988. " Role of the glutathione redox cycle in acquired and de novo multidrug resistance", *Science,* Vol.241, pp.694-697.

Laemmli, U. K., 1970. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". *Nature*, Vol.227, pp.680-685.

Lattes, R., 1980. " Lobular neoplasia (lobular carcinoma in situ) of the breast: A histological entity of controversial clinical significance" *Pathol. Res. Prat.*, Vol.166, pp.415.

Lawrence D. T., 1994. "Atlas of diagnostic immunohistopathology" J. B. Lippincott Company Philadelphia.

Lee,C.Y., Johnson,L., Cow, R.H., McKinney, J.D., and Lee, S.M., 1981. "Mouse liver GST". *J. Biol. Chem.*, Vol.256, pp.8110-8116.

Lee, W.-H., Morton, R. A., Epstein, J. I., Brooks, J. D., Campbell, P. A., Bova, G. S., Hsieh, W.-S., Isaacs, W. B., and Nelson, W. G., 1994. " Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis", *Proc. Natl. Acad. Sci. U.S.A.*, Vol.91, pp.11733-11737.

Leeson, T. S., Leeson, C. R., and Paparo, A. A., 1988. " Text/Atlas of Histology", Philadelphia, W. B. Saunders.

Legha, S. S., 1988. " Tamoxifen in the treatment of breast cancer", *Ann. Intern. Med.*, Vol.109, pp.219.

LeGal, M., Ollivier, L., Asselain, B., 1992. " Mammographic features of 455 invasive lobular carcinomas", *Radiology*, Vol.185, pp.705.

LeRoy, X., Excot, C., Brouillet, J. P., Theillet, C., Maudelonde, T., Simony-Lafontaine, J., Pujol, H., Rochefort, H., 1991. " Decrease of c-erbB-2 and c-myc RNA levels in tamoxifen-treated breast cancer", *Oncogene*, Vol.6, pp.431-437.

Leyland-Jones, B. R., Townsend, A. J., Tu, C. D., Cowan, K. H., Goldsmith, M. E.., 1991. " Antineoplastic drug sensitivity of human MCF-7 breast cancer cells stably transfected with a human alpha class glutathione-S-transferase gene", *Cancer Res.*, Vol.51, pp.587-594.

Lewis, A. D., Hickson, I. D., Robson, C. N., Harris, A. L., Hayes, J. D., Griffiths, S. A., Manson, M. M., Hall, A. E., Moss, J. E., Wolf, C. R., 1988. " Amplification and increased expression of alpha class glutathione S-transferase-encoding genes associated with resistance to nitrogen mustards", *Proc. Natl. Acad. Sci. USA*, Vol.85, pp.8511-8515.

Lewis, A.D., Forrester,L.M., Hayes, J.D., Wareing, C.J., Carmichael, J., Haris, A.L., Mooghen, M., and Wolf, C.R. 1989 "Glutathione S-transferase isoenzymes in human tumors and tumor derived cell lines" *Br. J. Cancer*. Vol.60, pp.327-331.

Liebau, E., Schonberger, O. L., Walter, R. D., and Henkle-Duhrsen, K., 1994. " Molecular cloning and expression of a cDNA encoding glutathione S-transferase from *Ascaris suum*", *Mol. Biochem. Parasitol.*, Vol.63, pp.167-170.

Lippman, M. E., 1983. " Antiestrogen therapy of breast cancer", *Semin. Oncol.*, Vol.10 (4 Suppl. 4), pp.11.

Liu, L.-F. and Tam, M. F., 1991. " Nucleotide sequence of a class mu glutathione S-transferase from chicken liver", *Biochim. Biophys. Acta.*, Vol.1090, pp.343-344.

Liu, Y. H., Taylor, J., Linko, P., Lucier, G. W., and Thompson, C. L., 1991. " Glutathione S-transferase mu in human lymphocyte and liver: role in modulating formation of carcinogen-derived DNA adducts" *Carcinogenesis*, Vol.12, pp.2269-2275.

Love, R. R., 1989. " Tamoxifen therapy in primary breast cancer: Biology, efficiancy, and side effects", *J. Clin. Oncol.*, Vol.7, pp.803.

Lyding, J., Damon, L., Wolf, J., 1992. " High-dose cyclophosphamide, thiotepa, and mitoxantrone with autologous bone marrow transplant for breast cancer", *Proc. A. S. C. O.*, Vol.11, pp.70.

Mannervik, B., 1985 "Glutathione peroxidase", *Methods Enzymol.*, Vol.113, pp. 490-495.

Mannervik, B., 1985. "The isoenzymes of glutathione S-transferase", *Adv. Enzymol. Rel. Areas Mol. Biol.*, Vol.57, pp.357-417.

Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M., and Jornvall, H., 1985. " Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties", *Proc. Natl. Acad. Sci. U.S.A.*, Vol.82, pp.7202-7206.

Mannervik, B.,1986. "Glutathione and the evolution of enzymes for the detoxication of products of oxidative metabolism", *Chem. Src.*, Vol.26B, pp.281-284.

Mannervik, B., Castro, V.M., Danielson, U.H., Tahir, M.K., Hansson, J., and Ringborg, U., 1987. "Expression of class Pi glutathione transferase in human malignant melanoma cells", *Carcinogenesis*, Vol.8, pp.1929-1932.

Mannervik, B. and Danielson, U. H., "Glutathione transferases- structure and catalytic activity", 1988. *Crit. Rev. Biochem. Mol. Biol.*, Vol. 23, pp.283-337.

Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Widersten, M., and Wolf, C. R., 1992. " Nomenclature for human glutathione transferases", *Biochem. J.*, Vol.282, pp.305-306.

Manni, A., Trujilo, J. E., Marshall, J. S., 1979. " Antihormone treatment of stage IV breast cancer", *Cancer, Vol.43, pp.444.*

Martinez, V., and Azzopardi, G., 1979. " Invasive lobular carcinoma of the breast: Incidence and variants", *Histopathology,* Vol.3, pp.467.

Masai, E., Katayama, Y., Kubota, S., Kawai, S., Yamasaki, M., and Morohoshi, N., 1993. " A bacterial enzyme degrading the model lignin compound β-etherase is a member of the glutathione S-transferase superfamily", *FEBS Lett.*, Vol.323 pp.135-140.

McGuire, W. L., 1986. " Prognostic factors in primary breast cancer", *Cancer Surveys,* Vol.5, pp.528.

McKay, J.A., Murray,G.I., Weaver, R.J., Ewen, S.W.B., Melvin, W.T., Burke, M., 1993. "Xenobiotic metabolizing enzyme expression in colonic neoplasia" *Gut*, Vol.34. pp.1234-1239.

McKee, P.H., Hobbs, C., Hall, P.A., 1993. "Antigen retrieval by microwave irradiation lowers immunohistological detection thresholds", *Histopathology*, Vol.23, No.4, pp.377-379.

McLennan, L.I., and Hayes, J.D., 1987. "Sex-specific constitutive expression of the pre-neoplastic marker glutathione transferase, YfYf, in mouse liver", *Biochem. J*, Vol.245, pp.399-406.

Mekhail-Ishak, K., Hudson, N., Tsao, M., Batist, G., 1989. "Implications for therapy of drug-metabolizing enzymes in human colon cancer" *Cancer Research.* Vol.49, pp.4866-4869.

Meyer, D.J., and Ketterer, B.,1982. "5 Alpha, 6 alpha-epoxy-cholestan-3 beta-ol (cholesterol alpha-oxide): a specific substrate for rat liver glutathione transferase B", *FEBS Lett.*, Vol.150, pp.499-502.

Meyer, D. J., Christodoulides, L.G., Tan, K.H., and Ketterer, B., 1984. "Isolation, prorerties and tissue distribution of rat glutathione transferase E", *FEBS Lett.*, Vol.173, pp. 327-330.

Meyer, D.J., Coles, B., Pemble, S.E., Gilmore, K.S., Fraser, G.M., and Ketterer, B., 1991." Theta, a new class of glutathione transferases purified from rat and man", *Biochem.J.*, Vol.274, pp.409-414.

Meyer, D. J. and Thomas, M., 1995. " Characterization of rat spleen prostaglandin-H D-isomerase as a sigma class GSH transferase", *Biochem. J.*, Vol.311, pp.739-742.

Miller, K., Auld, J., Jessup, E., Rhodes, A., Ashton-Key, M., 1995. "Antigen unmasking in formalin-fixed routinely processed paraffin wax-embedded sections by pressure cooking: a comparison with microwave oven heating and traditional methods", *Advances in Anatomical Pathology*, Vol.2, pp.60-64.

Miyazaki, M., Kohno, K., Saburi, Y., Matsuo, K., Ono, M., Kuwano, M., Tsuchida, S., Sato, K., Sakai, M., Muramatsu, M., 1990. " Drug-resistance to cis-diamminedichloroplatinum (II) in chinese hamster ovary cell-lines transfected with glutathione S-transferase pi gene", *Biochem. Biophys. Res. Commun.*, Vol.166, pp.1358-1364.

Moorghen, M., Cairns, J., Forrester, L.M., Hayes, J.D., Hall, A., Cattan, A.R., Wolf, C., and Harris, A.L., 1991. "Enhanced expression of glutathione S-transferases in colorectal carcinoma compared to non-neoplastic mucosa" *Carcinogenesis*. Vol.12 No.1. pp.13-17.

Morgenstern, R. and DePierre, J. W., 1983. " Microsomal glutathione transferase. Purification in unactivated form and further characterization of the activation process,

substrate specificity and amino acid composition", *Eur. J. Biochem.*, Vol.134, pp.591-597.

Morrow, C. S., Cowan, K. H., 1990. "Glutathione S-transferases and drug-resistance", *Cancer Cells*, Vol,2, pp.15-22.

Moscow, J. A., Townsend, A. J., Goldsmith, M. E., Whang-Peng, J., Vickers, P. J., Poisson, R., Legault-Poisson, S., Myers, C. E., and Cowan, K. H., 1988. " Isolation of the human anionic glutathione S-transferase cDNA and the relation of its gene expression to estrogen-receptor content in primary breast cancer", *Proc. Natl. Acad. Sci. U.S.A.*, Vol.85, pp.6518-6522.

Moscow, J. A., Townsend, A. J., Cowan, K. H., 1989. " Elevation of pi class glutathione S-transferase activity in human breast cancer cells by transfection of the GST pi gene and its effect on sensitivity to toxins", *Mol. Pharmacol.*, Vol.36, pp.22-28.

Moscow, J.A., Fairchild, C.R., Madden, M.J., 1989. "Expression of anionic glutathione S-transferase and P-glycoprotein genes in human tissues and tumors" *Cancer Research* Vol.49, pp.1422-1428.

Mozer, T.J., Tiemeier, D.C., and Jaworski, E.G., 1983. "Purification and characterization of corn glutathione S-transferase", *Biochemistry*, Vol.22, pp.1068-1072.

Mulder, T. P. J., Verspaget, H. W., Sier, C. F. M., Roelofs, H. M. J., Ganesh, S., Griffioen, G., and Peters, W. H. M., 1995. " Glutathione S-transferase pi in colorectal tumors is predictive for overall survival", *Cancer Res.*, Vol.55, pp.2696-2702.

Murphy, D., McGrown, A. T., Hall, A., Cattan, A., Crowther, D., and Fox, B. W., 1992. " Glutathione S-transferase activity and isoenzyme distribution in ovarian tumour

biopsies taken before or after cytotoxic chemotherapy", *Br. J. Cancer,* Vol.66, pp.937-942.

Nakachi K., Hayashi S-I, and Kawajiri, K., 1993. "Polymorphisms of the CYPIAI and glutathione S-transferase genes associated with susceptibility to lung cancer in relation to cigarette dose in a Japanese population" *Cancer Res.,* Vol.53. pp.2994-2999.

Nakagawa, K., Yokota, J., Wada, M., Sasaki, Y., Fujiwara, Y., Sakai, M., Muramatsu, M., Terasaki, T., Tsunokawa, Y., Terada, M., Sijo, N., 1988. " Levels of glutathione S-transferase-pi messenger-RNA in human-lung cancer cell-lines correlate with the resistance to cisplatin and carboplatin", *Jpn. J. Cancer Res.,* Vol.79, pp.301-304.

Nakagawa, K., Saijo, N., Tsuchida, S., Sakai, M., Tsunokawa, Y., Yokota, J., Muramatsu, M., Sato, K., Terada, M., and Tew, K. D., 1990. " Glutathione S-transferase pi as a determinant of drug resistance in transfectant cell lines", *J. Biol. Chem.,* Vol.265, pp.4296-4301.

Nazar-stewart, V., Motulsky, A. G., eaton, D. L., White, E., Hornung, S. K., Leng, Z.-T., Stapleton, P., and Weiss, N. S., 1993. " The glutathione S-transferase mu polymorphism as a marker for susceptibility to lung carcinoma", *Cancer Res.,* Vol.53, pp.2313-2318.

Nicholson, D. W., Ali, A., Vaillancourt, J. P., Calaycay, J. R., Mumford, R. A., Zamboni, R. J., and Ford-Hutchinson, 1993. " Purification to homogeneity and the N-terminal sequence of human leukotriene C_4 synthase: a homodimeric glutathione S-transferase composed of 18-kDa subunits", *Proc. Natl. Acad. Sci. U.S.A.,* Vol.90, pp.2015-2019.

Nicholson R. I., McClelland, R. A., Finlay, P., Eaton, C. L., Gullick, W. I., Dixon, A. R., Robertson, J. F. R., Ellis, I. O., Blamey, R. W., 1993. " Relationship between EGF-

R, c-erbB2 protein expression and Ki67 immunostaining in breast cancer and hormone sensitivity", *Eur. J. Cancer,* Vol.29A, pp.1018-1023.

Nimmo and Spalding, 1985. "The glutathione S-transferase activity in the kidney of rainbow trout (Salmo Gairdneri)"*Comp. Biochem Physiol.*, Vol.82B, No.1, pp.91-94.

Noguchi, M., Koyasaki, N., Ohta, N., 1993. " Internal mammary nodal status is a more reliable prognostic factor than DNA ploidy and c-erb B_2 expression in patients with breast cancer", *Arch. Surg.*, Vol.128, pp.242-246.

Nomura, Y., Tashiro, H., and Shinozuka, K., 1985. " Changes of steroid hormone receptor content by chemotherapy and endocrine therapy in anvanced breast cancer", *Cancer,* Vol.55, pp.546.

Norton, A. J., Jordan, S., Yeomans, P., 1994. "Brief, high-temperature heat denaturation (pressure cooking)-a simple and effective method of antigen retrieval for routinely processed tissues" *Jornal of Pathology,* Vol.173, No.4, pp.371-379.

Okuyama, T., Maehara, Y., Endo, K., Baba, H., Adachi, Y., Kuwano, M., and Sugimachi, K., 1994. " Expression of glutathione S-transferase-pi and sensitivity of human gastric cancer cells to cisplatin", *Cancer,* Vol.74, pp.1230-1236.

Orbo, A., Stalsberg, H., and Kunde, D., 1990. " Topographic criteria in the diagnosis of tumour emboli in intramammary lymphatics", *Cancer,* Vol.66, pp.972.

Oshino, R., Kamei, K., Nishioka, M., and Shin, M., 1990. "Purification and characterization of glutathione S-transferases from guinea pig liver", *J. Biochem.*, Vol.107, pp.105-110.

Pabst, M.J., Habig, W.H., and Jakoby, W. B., 1973. " Mercapturic acid formation: the several glutathione transferases of rat liver", *Biochem. Biophys. Res. Commun.*, Vol.52, pp.. 1123-1128.

Pacifici, G.M., Warholm, M., Gutenberg, C., Mannervik, B., and Rane, A., 1986. "Organ distribution of glutathione transferase isoenzymes in the human fetus: differences between liver and extrahepatic tissues" *Biochem. Pharmacol.*, Vol.35, pp.1616-1619.

Page, D. L., and Anderson, T. J., 1987. " Diagnostic Histopathology of the Breast ", New York, Churchill Livingston, pp.198-205.

Pemble, S. E. and Taylor, J. B., 1992. " An evolutionary perspective on glutathione transferases inferred from class-theta glutathione transferase cDNA sequences", *Biochem. J.*, Vol.287, pp.957-963.

Pemble, S., Schroeder, K. R., Spencer, S. R., Meyer, D. J., Hallier, E., Bolt, H. M., Ketterer, B., and Taylor, J. B., 1994. " Human glutathione-S-transferase Theta (GSTT1):cDNA cloning and the characterization of a genetic polymorphism", *Biochem. J.*, Vol.300, pp.271-276.

Perry, R. R., Mazetta, J., Levin, M., Barranco, S. C., 1993. " Glutathione levels and variability in breast tumours and normal tissue", *Cancer*, Vol.72, pp.783-787.

Peters, W. H. M., Nagengast, F. M., and Wobbes, T., 1989." Glutathione S-transferases in normal and cancerous human colon tissue" *Carcinogenesis* Vol.10, No.12, pp.2371-2374.

Peters, W.H.M., Wormskamp, N.G.M., and Thies, E., 1990.. "Expression of glutathione S-transferases in normal gastric mucosa and in gastric tumors" *Carcinogenesis* Vol.11 No.9, pp.1593-1596.

Peters, H.H.M., Wobbes, T., Roelofs, H.M.J. and Jansen, J.B.M., 1993 "Glutathione S-transferases in esophageal cancer" *Carcinogenesis* Vol.14, No.7, pp.1377-1380.

Pickett, C. B., Telakowski-Hopkins, C. A., Argenbright, L, and Lu, A. Y. H., 1984. " Regulation of glutathione S-transferase mRNAs by phenobarbital and 3-methylcholanthrene: analysis using cDNA probes", *Biochem. Soc. Trans.*, Vol. 12, pp.71-74.

Pickett, C. B., 1989. "Glutathione S-transferases: gene structure, regulation, and biological function". *Ann. Rev. Biochem.*, Vol.58, pp.743-764.

Ploemen, J. H. T. M., Wormhoudt, L. W., van Ommen, B., Commandeur, J. N. M., Vermeulen, V. P. E., and van Bladeren, P. J., 1995. " Polymorphism in the glutathione conjugation activity of human erythrocytes towards ethylene dibromide and 1,2-epoxy-3-(p-nitrophenoxy)propane", *Biochim. Biophys. Acta,* Vol.1243, pp. 469-476.

Polidoro, G., Di Illio, C., Del Bccio, G., Zulli, P., and Federici, G., 1980. "Glutathione S-transferase activity in human placenta", *Biochem. Pharmacol.*, Vol.29, pp.1677-1680.

Puchalski, R. B., Fahl, W. E., 1990. " Expression of recombinant glutathione S-transferase pi, Ya, or Yb1 confers resistance to alkylating agents", *Proc. Natl. Acad. Sci. USA,* Vol.87, pp.2443-2447.

Raam, S., Robert, N., Pappas, C. A., 1988. " Defective estrogen receptors in human mammary cancers: Their significance in defining hormone dependence", *J. Natl. Cancer Inst.*, Vol.80, pp.756.

Radulovic, L.L., Kulkarni, A.D., and dauterman, W.C., 1987. "Biotransformation of methyl parathion by human foetal liver glutathione S-transferases: an in vitro study ", *Xenobiotica*, Vol.17, pp. 105-114.

Ranganathan, S., Tew, K., 1991 "Immunohistochemical localization of glutathione S-transferases alpha, mu, and pi in normal tissue and carcinomas from human colon" *Carcinogenesis* Vol.12, No.12, pp.2383-2387.

Raunio H., Husgafvel-Pursiainen K., 1995. "Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility" *Gene* Vol.159.pp.113-121.

Ravdin, P. M., Green, S., Dorr, T. M., 1992. " Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: Results of a prospective Southeast Oncology Group study", *J. Clin. Oncol.*, Vol.10, pp.1284.

Reddy, C.C., Burgess, J.R., Gong, Z.Z., Massaro, E.J., and Tu, C.P.D., 1983. "Purification and characterization of the individual glutathione S- transferases from sheep liver ", *Arch. Biochem. Biophys.*, Vol.224, pp.87-101.

Redick, J. A., Jakoby, W. B., and Baron, J., 1982. "Immunohistochemical localization of glutathione S-transferases in liver of untreated rats" *J. Biol.Chem.*, Vol.257, pp.15200-15203.

Reed, D.J., and Fariss, M.W., 1984. "Glutathione deplation and susceptibility", *Pharmacol. Rev.*, Vol.36, pp.25S-33S.

Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gallay, O., and Huber, R., 1991. " The three-dimensional structure of class pi glutathione-S-transferase in complex with glutathione sulfonate at 2.3 A resolution" *EMBO J.*, Vol. 10, pp.1997-2005.

Rilke, F., Colnaghi, M. I., Cascinelli, N., 1991. " Prognostic significance of HER-2/neu expression in breast cancer and its relationship to other prognostic factors", *Int. J. Cancer*, Vol.49, pp.44.

Robertson, I.G.C., Guthenberg, C., Mannervik, B., and Jernstrom, B.,1986. "Differences in stereoselectivity and catalytic efficiency of three human glutathione transferases in the conjugation of glutathione with 7 beta, 8 alpha-dihydroxy-9alpha 10 alpha-oxy-7,8,9,10 tetrahydro benzo(a)pyrene", *Cancer Res.*, Vol.46, pp.2220-2224.

Robson, C. N., Lewis, A. D., Wolf, C. R., Hayes, J. D., Hall, A., Proctor, S. J., Harris, A. L., and Hickson, I. D., 1987. " Reduced levels of drug-induced DNA cross-linking in nitrogen mustard-resistant Chinese hamster ovary cells expressing elevated glutathione S-transferase activity", *Cancer Res.*, Vol.47, pp.6022-6027.

Rosen, P. P., and Oberman, H. A., 1992. " Atlas of Tumour Pathology. Tumours of the Mammary Gland", Washihgton, D. C., Armed Forces Institute of Pathology.

Rosner, D., Bedwani, R. N., Vana, J., 1980. " Noninvasive breast carcinoma. Results of a national survey by the American College of Surgeons", *Ann. Surg.*, Vol.192, pp.139.

Ross, M. H., Reith, E. J., and Romrell, L. J., 1988. " Histology: A text and Atlas", Baltimore, Williams & Wilkins.

Ross, V. L., Board, P. G., and Webb, G. C., 1993. " Chromosomal mapping of the human mu class glutathione S-transferases to 1p13", *Genomics*, Vol.18, pp.87-91.

Safa, A. R., Glover, C. J., Meyers, M. B., Biedler J. L., Felsted, R. L., 1986. " Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells", *J. Biol. Chem.*, Vol.261, pp.6137-6140.

Saneto, R.P., Awasthi, Y.C., and Srivastava, S.K.,1980."Interrelationship between cationic and anionic forms of glutathione S-transferases of bovine ocular lens", *Biochem.J.*, Vol.191, pp. 11-20.

Saxena, M., Allameh, A., Mukerji, K. G., and Raj, H. G., 1991. " Epoxidation of aflatoxin B_1 by *Aspergillus Flavus* microsomes in vitro: interaction with DNA and formation of aflatoxin B_1-glutathione conjugate, *Chem.-Biol. Interactions*, Vol.78, pp.13-12.

Seidegard, J., and Pero, R.W. 1985 " The hereditary transmission of high glutathione transferase activity toward transtilbene oxide in human mononuclear leucocytes" *Hum. Genet.*, Vol.69. pp. 66-68.

Seidegard, J., Pero, R.W., Miller, D.G., and Beattie, E.J., 1986. "A glutathione transferase in human leucocytes as a marker for the susceptibility to lung cancer", *Carcinogenesis*, Vol.7, pp. 751-753.

Seidegard, J., Vorachek, W. R., Pero, R. W., and Pearson, W. R., 1988. " Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion", *Proc. Natl. Acad. Sci. U.S.A.*, Vol.85, pp.7293-7297.

Seidegard, J., Pero, R.W., Markowitz, M. M., Roush, G., Miller, D.G., and Beattie, E. J., 1990. "Isoenzymes of glutathione transferase (class mu) as a marker for the susceptibility to lung cancer". *Carcinogenesis* Vol 11 No.1 pp. 33-36.

Shea, T.C., Kelley,S.L., Henner,W.D., 1988 "Identification of an anionic form of glutathione transferase present in many human tumors and human tumor cell lines" *Cancer Research* Vol.48 pp.527-533.

Shea, T. C., Caflin, G., Comstock, K. E., 1990. " Glutathione S-transferase activity and isoenzyme composition in primary human breast cancers", *Cancer Res.*, Vol.50, pp.6848-6853.

Sheehan, D. and Casey, J. P., 1993. " Evidence for alpha and mu class glutathione S-transferases in a number of fungal species", *Comp. Biochem. Physiol.*, Vol.104B, pp.7-13.

Sherman, M., Titmuss, S., and Kirsch, R.E., 1983. " Glutathione S-transferase in human organs", *Biochem. Int.* Vol.6, pp.109-118

Shi, E.C.P., Fisher, R., McEvoy M., Vantol, R., Rose, M., and Ham, J.M., 1982. "Factors influencing hepatic glutathione concentrations -A study in surgical patients", *Clinical Science*, Vol.62, pp.279-283.

Shi, S. R., Key, M.E., Kalra, K.I., 1991. "Antigen retrieval in formalin-fixed, paraffin-embedded tissues-an enhancement method for immunohistochemical staining based on microwave-oven heating of tissue-sections", *Journal of histochemistry and cytochemistry*, Vol.39, No.6, pp.741-748.

Shields, P. G., Bowman, E. D., Harrington, A. M., Doan, V. T., and weston, A., 1993. "Polycyclic aromatic hydrocarbon-DNA adducts in human lung and cancer susceptibility genes", *Cancer Res.*, Vol.53, pp.3486-3492.

Shimada T., Hayes C.L., Yamazaki H., 1996. "Activation of chemically diverse procarcinogens by human cytochrome P450 IBI". *Cancer Research. Vol.56 pp.2979-2984.*

Shiratori, Y., Soma, Y., Maruyama,H., Sato, S., Atsushi T., Sato,K.,1987 "Immunohistochemical detection of the placental form of glutathione S-transferase in dysplastic and neoplastic human uterine cervix lesions" *Cancer Research* Vol.47, pp.6806-6809.

Simons, P.C., and Vander Jagt, P.L., 1977. "Purification of glutathione S-transferases from human liver by glutathione-affinity Chromatography", *Anal. Biochem.*, Vol.82, pp.334-341.

Singh, S.V., Haque, A.K., Ahmad, H., Awasthi, Y.C., 1988. " Glutathione S-transferase isoenzymes in human lung tumors" *Carcinogenesis* Vol.9 No. 9 pp. 1681-1685

Singh, S.V., Roberts, B., Gudi, V.A., Ruiz, P., and Awasthi, Y., 1991. "Immunohistochemical localization, purification, and characterization of human urinary bladder glutathione S-transferases" *Biochimica et Biophysica Acta* Vol.1074, pp.363-370.

Singhal, S. S., Zimniak, P., Sharma, R., Srivastava, S. K., Awasthi, S., and Awasthi, Y. C., 1994. " A novel glutathione S-transferase isoenzyme similar to GST 8-8 of rat and mGSTA4-4 (GST 5.7) of mouse is selectively expressed in human tissues", *Biochim. Biophys. Acta.*, Vol.1204, pp.279-286.

Sinha, B. K., Katki, A. G., Batist, G., Cowan, K. H., Myers, C. E., 1987. " Differential formation of hydroxyl radicals by adriamycin in sensitive and resistant MCF-7 human breast tumor cells: Implications for the mechanism of action", *Biochemistry,* Vol.26, pp.3776-3781.

Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. L., Armstrong, R. N., Ji, X., Board, P. G., Olin, B., Mannervik, B., and Jones, T. A., 1993. " Structure determination and refinement of human alpha class glutathione transferase A1-1, and a comparison with the mu and pi class enzymes", *J. Mol. Biol.*, Vol.232, pp.192-212.

Smith, G.J., Ohl, V.S., and Litwack, G., 1980. " Purification and properties of hamster liver ligandins, Glutathione S-transferases", *Cancer Res.*, Vol. 40, pp.1787-1790.

Smith, M. J., Evans, C. G., Doane-Setzer, P., Castro, V. M., Tahir, M. K., and Mannervik, B., 1989. " Denitrosation of 1,3-Bis(2-chloroethyl)-1-nitrosourea by class mu glutathione transferases and its role in cellular resistance in rat brain tumor cells", *Cancer Res.*, Vol.49, pp.2621-2625.

Snell, R. S., 1984. " Clinical and Functional histology for Medical Students", Boston, Little, Brown & Co.

Somfai-Relle, S., Suzukake, K., Vistica, B. P., Vistica, D. T., 1984. "Reduction of cellular glutathione by buthionine sulfoximine and sensitization of murine tumor-cells resistant to L-phenylalanine mustard", *Biochem, Pharmacol.*, Vol.33, pp.485-490.

Soma, Y., Satoh, K., Sato, K., 1986. " Purification and subunit-structural and immunological characterization of five glutathione S-transferases in human liver, and the acidic form as a hepatic tumor marker", *Biochim. Biophys. Acta.*, Vol.869, pp.247-258.

Soubeyran, I., Quenel, N., Mauriac, L., Duran, M., Bonichon, F., Coindre, J-M., 1996. " Variation of hormonal receptor, pS2, c-erbB-2 and GST pi contents in breast carcinomas under tamoxifen: a study of 74 cases", *Br. J. Cancer,* Vol.73, pp.735-743.

Spratt, J. S., 1994. " Realities of breast cancer control, public expectations and the law", *Surg. Oncol. Clin. North. Am.*, Vol.3, pp.25-44.

Srivastava, P. K., and Waxman, D. J., 1993. " Sex-dependent expression and growth hormone regulation of class alpha and class mu glutathione S-transferase mRNAs in adult rat liver", *Biochem. J.*, Vol.294, pp.159-165.

Staats, J., Marquardt, D., Center, M. S., 1990. "Characterization of a membrane-associated protein-kinase of multidrug-resistant HL60 cells which phosphorylates P-glycoprotein" *J. Biol. Chem.*, Vol.265, pp.4084-4090.

Stenersen, J., Kobro, S., Bjerke, M., and Arend, U., 1987. " Glutathione transferases in aquatic and terrestrial animals from nine phyla", *Comp. Biochem. Physiol.*, Vol86C, pp.73-82.

Sternberger, LA, 1978. "Immunocytochemistry", Englewood Cliffs, Prentice-Hall.

Stewart, V.N., Motulsky, A.G., Eaton, D.L., White, E., 1993. "The glutathione S-transferase mu polymorphism as a marker for susceptibility to lung carcinoma" *Cancer Research* Vol. 53, pp.2313-2318.

Stockman, P. K., Beckett, G. J., and Hayes, J. D., 1985. " Identification of a basic hybrid glutathione S-transferase from human liver. Glutathione S-transferase δ is composed of two distinct subunits (B1 and B2)", *Biochem, J.*, Vol.227, pp.457-465.

Stohs, S.J., Al-Turk, W.A., and Angle, C.R., 1982. "Glutathione S-transferase and glutathione reductase activities in hepatic and extra-hepatic tissues of female mice as a function of age", *Biochem. Pharmacol.*, Vol.31, pp.2113-2116.

Strange R.C., Faulder C.G., Davis B.A., 1984. "The human glutathione S-transferases: studies on tissue distribution and genetic variation of the GST1, GST2, and GST3 isoenzymes" *Ann. Hum. Genet.* Vol.48. pp.11-20.

Strange, R.C., Davis, B.A., Faulder, C.G., Cotton, W., Bain, A.D., Hopkinson, D.A., and Hume,R., 1985. "The human GST developmental aspects of the GST1, GST2, and GST3 loci", *Biochem. Genet.*, Vol.23, pp.1011-1028.

Strange, R. C., Matharoo, B., Faulder, G. C., Jones, P., Cotton, W., Elder, J. B., and *Deakin*, M., 1991. " The human glutathione S-transferases: a case-control study of the incidence of the GST1 0 phenotype in patients with adenocarcinoma", *Carcinogenesis*, Vol.12, pp.25-28

Strange, R.C., Fryer, A.A., Matharoo, B., Zhao, L., Broome, J., Campbell, Jones, P., Pastor, I. J., 1992. "The human glutathione S-transferase comparison of isoenzyme expression in normal and astrocytoma brain" *Biophys. Acta.* Vol.1139. pp.222-228.

Strenberger, L. A., Hardy, P. H., 1970. "The unlabeled antibody enzyme method of immunohistochemistry" *The Journal Histochem.* Vol.18, No.5 pp.315-333.

Sugiyama, Y., Tamada, T., and Kaplowitz, N., 1981 " GST in elasmobranch liver-molecular heterogeneity, catalytic and binding properties, and purification". *Biochem. J.*, Vol.199, pp.749-756.

Su-Ming Hsu, L. Raine, and H. Fanger.1981. "Use of Avidin-Biotin-Peroxidase Complex(ABC) in Immunoperoxidase Techniques" *The Journal of Histochem. and Cytochem.*, Vol.29, No.4, pp. 577-580.

Suzuki, T., Johnston, P. N., and Board, P. G., 1993. " Structure and organization of the human alpha class glutathione S-transferase genes and related pseudogenes", *Genomics*, Vol.18, pp.680-686.

Tahir, M. K., and Mannervik, B., 1986. " Simple inhibition studies for distinction between homodimeric and heterodimeric isoenzymes of glutathione transferase", *J. Biol. Chem.*, Vol.261, pp.1048-1051.

Talalay, P., De Long, M. J., and Prochaska, H. J., 1988. " Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis", *Proc. Natl. Acad. Sci. U.S.A.*, Vol.85, pp.8261-8265.

Tamaki, H., Kumagai, H., and Tochikura, T., 1990. " Glutathione S-transferase in yeast: induction of mRNA, cDNA cloning and expression in *Escherichia coli*", *Biochem. Biophys. Res. Commun.*, Vol.172, pp.669-675.

Tan, K. L., Webb, G. C., Baker, R. T., and Board, P. G., 1995. " Molecular cloning of a cDNA and chromosomal localization of a human theta-class glutathione S-transferase gene (GSTT2) to chromosome 22", *Genomics*, Vol.25, pp.381-387.

Tartter, P. I., Papatestas, A. E., Iannovich, L., 1981. " Cholesterol and obesity as prognostic factors in breast cancer", *Cancer*, Vol.47, pp.2222.

Taylor, R. E., Powles, T. J., Humphreys, J., Bettelheim, R., Dowsett, M., Casey, A. J., Neville, A. M., Coombes, R. C., 1982. " Effects of endocrine therapy on steroid receptor content of breast cancer", *Br. J. Cancer*, Vol.45, pp.80-85.

Taylor, C.R., Shi, S.R., Imam, S.A., Young, L., Cote, R.J., 1995. "Antigen retrieval immunohistochemistry under the influence of pH using monoclonal-antibodies", *Journal of Histochemistry and Cytochemistry*, Vol.43, No.2, pp.193-201.

Teicher, B.A., Holden, S.A., Kelley, M.J., Shea, T.C., Cucch, C.A., Rosowsky, A., Henner, W.D., and Frei, E., 1987. "Characterization of cis-diamine-dichloroplatinum(II)", *Cancer Res.*, Vol.47, pp.338-393

Tew, K. C., Bomber, A. W., Hoffman, S. J., 1988. " Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines", *Cancer Res.*, Vol.48, pp.3622-3625.

Tinnemans, J. G. M., Beex, L. V. A. M., Wobbes, Th., 1990. " Steroid hormone receptors in nonpalpable and more advanced stages of breast cancer. A contribution to the biology and natural history of carcinoma of the female breast", *Cancer*, Vol.66, pp.1165.

Tomorev, S. I. and Zinovieva, R. D., 1988. " Squid major lens polypeptides are homologous to glutathione S-transferase subunits", *Nature*, Vol.336, pp.86-88.

Towbin, H., Staehelin, T., and Gordon, J. 1979 "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications" *Proc. Natl. Acad. Sci. USA.*, Vol.76, pp. 4350-4354.

Townsend, A. J., Cowan, K. H., 1989. *Cancer Bull.*, Vol.41, pp.31-37.

Tsuchida, S., Maki, T., and Sato, K., 1990. " Purification and characterization of glutathione transferases with an activity towards nitroglycerin from human aorta and heart: multiplicity of the human mu forms", *J. Biol. Chem.*, Vol.265, pp.7150-7157.

Underwood, J. C. E., 1996. " General and Systemic Pathology", Churchil Livingstone.

Ünsal, I., and Ögüs, H., 1991. "Koyun karacigeri glutatyon trasferaz izozimlerinin saflastirilmasi", *Biyokimya Dergisi*, Vol.16, pp.27-42.

Wareing, C. J., 1990. " The Role of the Glutathione S-Transferases in Resistance to Cytotoxic Compounds", Ph. D. thesis, University of Edinburgh.

Warholm, M., Guthenberg, C., Mannervik, B., von Bahr, C., and Glaumann, H., 1980. " Identification of a new glutathione S-transferase in human liver", *Acta Chem. Scand. Scr. B.*, Vol.B34, pp.607-610.

Warholm, M., Guthenberg, C., Mannervik, B., Pacifici, G.M., and Rane, A., 1981b. " Glutathione S-transferases in human fetal liver", *Acta Chem. Scand. Ser. B.*, Vol.B35, pp.225-227.

Warholm, M., Guthenberg, C., Mannervik, B., 1983. "Molecular and catalytic properties of glutathione transferase mu from human liver: an enzyme efficiently conjugating epoxides", *Biochemistry*, Vol.22, pp. 3610-3617.

Warwick, A., Sarhanis, P., Redman, C., Pemble, S., Taylor, J. B., Ketterer, B., Jones, P., Alldersea, J., Gilford, J., Yengi, L., Fryer, A., and Strange, R. C., 1994. " Theta class glutathione S-transferase GSTT1 genotypes and susceptibility to cervical neoplasia: interactions with GSTM1, CYP2D6 and smoking", *Carcinogenesis*, Vol.15, pp.2841-2845.

Waseda, N., Kato, Y., Imura, H., Kurata, M., 1981. " Effects of tamoxifen on oestrogen and progesterone receptors in human breast cancer", *Cancer Res.*, Vol.41, pp.1984-1988.

Weidner, N., Semple, J. P., Welch, W. R., 1991. " Tumour angiogenesis and metastasis-correlation in invasive breast carcinoma" *N. Engl. J. Med.*, Vol.324, pp.1.

Weigand, R. A., Isenberg, W. M., Russo, J. 1982. " Blood vessel invasion and axillary lymph node involvement as prognostic indicators for human breast cancer", *Cancer,* Vol.50, pp.962.

Welchor, J. C., Rodriguez-Escudero, F. J., Lujan, S., Corcostegui, B., 1990. " Variation of oestrogen and progesterone receptor status in breast cancer after tamoxifen therapy", *Oncology,* Vol.47, pp.467-470.

Wellings, S. R., Jensen, H. M., and Marcum, R. G., 1974. " Atlas of subgross pathology of the human breast with special reference to possible precancerous lesions" *J. Natl. Cancer Inst.,* Vol.55, pp.231.

Widersten, M., Pearson, W. R., Engstrom, A., and Mannervik, B., 1991. "Heterologous expression of the allelic variant Mu-class glutathione transferases μ and ψ", *Biochem. J.,* Vol.276, pp.519-524.

Wiencke, J. K., Kelsey, K. T., Lamela, R. A., and Toscano, W. A., Jr., 1990. " Human glutathione S-transferase deficiency as a marker of susceptibility to epoxide-induced cytogenetic damage", *Cancer Res.,* Vol.50, pp.1585-1590.

Wiencke, J. K., Pemble, S., Ketterer, B., and kelsey, K. T., 1995. " Gene deletion of glutathione S-transferase theta-correlation with induced genetic damage and potential role in endogenous mutagenesis", *Cancer Epidemiol. Biomarkers Prev.,* Vol.4, pp.253-259.

Wilce, M. C. J., Board, P. G., Feil, S. C., and Parker, M. W., 1995. " Crystal structure of a theta-class glutathione transferase", *EMBO J.,* Vol. 14, pp.2133-2143.

William, L. D., Spratt, J. S., 1995. " Cancer of the Breast", W. B. Saunders Company.

Williams, M. T., Carrington, H., and Herrera, A., 1986. "Stimulation of mouse liver glutathione S-transferase activity in propylthiouracil-treated mice in vivo by triiodothyronine", *Biochem. J.*, Vol.233, pp.595-598.

Wilson, Y. G., Rhodes, M., Ibrahim, N. B. N., Padfield, C. J. H., Cowthorn, S. J., 1994. " Immunocytochemical staining of pS2 protein in fine-needle aspirate from breast cancer is an accurate guide to response to tamoxifen in patients aged over 70 years", *Br. J. Surg.*, Vol.81, pp.1155-1158.

Wittliff, J. L., 1984. "Steroid-hormone receptors in breast cancer", *Cancer*, Vol.53, pp.630.

Wolf, C. R., Hayward, I. P., Lawrie, S. S., Buckton, K., McIntyre, M. A., Adams, D. J., Lewis Scott, A. D., Scott, A. R., Smyth, J. F., 1987. " Cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient", *Int. J. Cancer*, Vol.39, pp.695-702.

Wolf, C. R., Wareing, C. J., Black, S. M., 1990. " Glutathione S-transferases in resistance to chemotherapeutic agents", Hayes J. D., Pickett, C. B., Mantle, J. J., (eds): Glutathione S-transferases and Drug Resistance. London, England, Taylor Francis, pp.295-306.

Wright, C., Nicholson, S., Angus, B., Sainsbury, J. R. C., Farndon, J., Cairns, J., Harris, A. L., Horne, C. H. W., 1992. " Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer", *Br. J. Cancer*, Vol.65, pp.118-121.

van Bladeren, P. J., van der Gen, A., Breimer, D. D., and Mohn, G. R., 1979. " Stereoselective activation of vicinal dihalogen compounds by glutathione conjugation" *Biochem. Pharmacol.*, Vol.28, pp.2521-2524.

Vos, R. M. E. , and Bladeren, 1990. "Glutathione S-transferases in relation to their role in the biotransformation of xenobiotics", *Chem. Biol. Interactions*, Vol.75, pp.241-265.

Yang, W. Z., Begleiter, A., Johnston, J. B., Israels, L. G., and Mowat, M. R. A., 1992. " Role of glutathione and glutathione S-transferase in chlorambucil resistance", *Mol. Pharmacol.*, Vol.41, pp.625-630.

Yeung, T.C., and Gidari, A.S., 1980. "Purification and properties of a chicken liver glutathione S-transferase", *Arch. Biochem. Biophys.*, Vol.205, pp.404-411.

Yengi L., Inskip A., GiffordJ., 1996. "Polymorphism at the glutathione S-transferase locus interactions with cytochrome P450 and the glutathione S-transferase genotypes factors for multiple cutaneous basal cell carcinoma" *Cancer Research* Vol.56 pp.1974

Zhong, S., Howie, A. f., Ketterer, B., taylor, J. B., Hayes, J. D., Beckett, G. J., Wathen, C. G., Wolf, C. R., and Spurr, N. K., 1991. " Glutathione S-transferase mu locus: use of genotyping and phenotyping assays to assess association with lung cancer susceptibility", *Carcinogenesis*, Vol.12, pp.1533-1537.

APPENDIX A

PREPARATION OF IMMUNOHISTOCHEMICAL STAINING SOLUTIONS

Preparation of APES Slides:

-Take clean slides to tap water
-Transfer to hot, soapy water
-Rinse very well in running tap water
-Rinse in distilled water
-Dry in slide dryer (approximately 30 min)
-Dip in 2% APES solution (Sigma) in acetone
-Allow to drain, but not fully dry (slide goes grainy)
-Rinse in running tap water
-Rinse in distilled water
-Dry and return to clean slide box

Antigen Retrieval by Wet Autoclave Pre-treatment Procedure (Pressure Cooking):

-Place 1.6 l of 0.01M citrate buffer pH 6.0 in a pressure cooker
-Switch hotplate onto highest temperature
-While buffer is boiling take sections to water
-When the buffer is boiling add the sections in metal slide racks
-Seal lid on correctly and put weight onto 1 dot position
-When timer behind weight lifts start timing for 3 min (time may differ)
-After 3 min switch weight to 2 dot position

-Take off the heat and place in running cold water

-Place sections in warm water

-Transfer sections to TBS for immunohistochemistry

Strept/ABC HRP (kit method) Solution:

0.05 M TBS buffer	5 ml
A (Bio-HRP)	45µl
B (streptavidin)	45µl

mix in the written order and leave this solution for 20 min at 0-4^{0}C before using.

DAB(diaminobenzidine) Solution:

TBS buffer	20 ml
30% H_2O_2 soln.	200µl
Diluted DAB (10 mg/ml)	1 ml

mix in the written order and leave this solution for 10 min at 0-4^{0}C before using.

Harris Hematoxylin Solution:

Hematoxylin	5 g
Ethanol	50 ml
Potassium Ammonium Alum	100 g
Distilled water	1 litre
Mercuric oxide 2.5 g or Sodium iodate	1 g
Glacial Acetic Acid	40 ml

Eosin Solution (1% Aqueous Eosin in 0.1% Calcium Nitrate):

Calcium Nitrate	1 g
Distilled Water	1 litre
Eosin Y Powder	10 g

1% Acid alcohol Solution:

70% alcohol	99 ml
Concentrated HCI	1 ml

0.05 M TBS (Tris buffered saline), pH 7.6 (10X TBS):

Tris base	50 mM
Sodium chloride	150 mM
Adjust pH to 7.6 with 1 M HCI	

0.005 M TBS (Tris buffered saline), pH 7.6 (1X TBS):

Tris base	5 mM
Sodium chloride	15 mM
Adjust pH to 7.6 with 1 M HCI	

10X 0.05 M TBS (Tris buffered saline), pH 7.6 :

Tris base	60.55 g
Sodium chloride	85.20 g
Distilled water	500 ml

Adjust pH to 7.6 with 370 ml 1 M HCI and made up to 1 litre.
(Dilute 1:10 before using)

Antigen Retrieval Solution, (0.01 M Buffered Sodium Citrate Solution), pH 6.0 :

10X 0.01 M sodium citrate (B)

Sodium citrate	14.7 g
Distilled water	500 ml

10X 0.01 M citric acid (A)

Citric acid	2.101 g
Distilled water	100 ml

Antigen retrieval solution (0.01 M Buffered Sodium Citrate Solution), pH 6.0

Solution A	9 ml
Solution B	41 ml
Distilled water	450 ml

4% Paraformaldehyde (10% formalin), pH 6-8 :

100% formalin or 37-40% formaldehyde	100 ml
Distilled water	900 ml

TED Buffer (10mM Tris-HCI buffer), pH 7.8 :

Tris	10mM
EDTA	1mM
DTT	1mM
PMSF	100μM

Adjust the pH of the solution to 7.8 by adding 0.1 M HCI and complete the solution to 1 litre with distilled water.

APPENDIX B

WHO HISTOLOGIC CLASSIFICATION OF BREAST TUMOURS (BLOOM AND RICHARDSON)

Grade I: 3-5 points

Grade II: 6-7 points

Grade III: 8-9 points

Add scores for each component

Tubule formation

1. Majority of tumour (>75%)
2. Moderate (10-75%)
3. Little or none (<10%)

Nuclear pleomorphism

1. Regular, uniform
2. Larger with variation
3. Marked variation

Mitoses

Number of mitoses per 10 hpf at periphery of tumour. Hyperchromatic figures are not included.

Mit per 10 hpf	Score
>16	3
8-15	2
0-7	1

CURRICULUM VITAE

Serpil (Aydın) Oğuztüzün was born in İskenderun, Turkey on April 10, 1968. She received B.S. in Science Education in July 1990, and M.S. in Science Education in January 1993, both from the Middle East Technical University. She has worked as a science teacher in secondary education since 1991. She has participated in several conferences and workshops. She performed research at Liverpool John Moores University School of Biomolecular Sciences between October 1997 and October 1998 as a British Council Chevening Scholar. Her main areas of interest are toxicology and cancer particularly enzyme localization by immunohistochemical technique and specific enzymes expression and alteration in tumours and cancer therapy.

Publications

Bayraktaroğlu, E. and Aydın, S., Neurosecretory Cells in Buccal Ganglia of *Helix pomatia*. (*Poster*) *Second National Congress on Medical Biology*, Hacettepe University, Ankara, Turkey, September 1992.

Bayraktaroğlu, E. and Aydın, S., Neurosecretory Cells in Buccal Ganglia of *Helix Pomatia*. (*Poster*) *17th Conference of European Comparative Endocrinologists*, Department of Cell Biology, University of Cordoba, Spain, 5-10 September, 1994.