

The Role of Glutaminase in Cancer

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Abstract

Increased glutamine metabolism (glutaminolysis) is a hallmark of cancer and is recognised as a key metabolic change in cancer cells. As a heterogeneous disease with different morphological and molecular subtypes and response to therapy, breast cancer cells are known to rewire glutamine metabolism to support survival and proliferation. Glutaminase isoenzymes (GLS and GLS2) are key enzymes for glutamine metabolism. Interestingly, GLS and GLS2 display contrasting functions in tumourigenesis. In this review, we explore the role of glutaminase in cancer, primarily focussing on breast cancer, address the role played by oncogenes and tumour suppressor genes in regulating glutaminase, and discuss current therapeutic approaches in targeting glutaminase.

Keywords: glutaminase, glutamine, metabolism, cancer, breast cancer, subtypes

Introduction

An established hallmark of cancer is undergoing metabolic reprogramming to maintain high demand for energy needed to sustain proliferation and survival [1]. Cancer cells alter their glucose and glutamine metabolism to acquire sufficient energy and cellular building blocks needed to support this unremitting growth. Some consume more glucose showing aerobic glycolysis or the ‘Warburg effect’, where glucose is converted mainly to lactic acid instead of engaging in the mitochondrial oxidative phosphorylation to allow proper respiration [2-4]. Other cancer cell types fail to grow or proliferate in the absence of glutamine and display ‘glutamine addiction’ [5], which helps the cells to sustain high proliferative rates under conditions of hypoxia and glucose depletion [6-8] (Figure 1).

The intracellular processing of glutamine begins with its catalysis by glutaminase. In this review we discuss the role, regulation and relevance of glutaminase and its isozymes and splice variants in cancer particularly focussing on breast cancer (BC). We further highlight the opportunities that exist in utilising glutaminase as a therapeutic target.

Glutamine metabolism and addiction in cancer

Glutamine metabolism plays an important role in normal cell metabolism and generating energy for the rapidly proliferating cells and tissues. As the most abundant amino acid in blood circulation, glutamine serves directly or indirectly, via its metabolic products glutamate and alpha ketoglutarate, as carbon and nitrogen sources needed for the biosynthesis of nucleic acids, fatty acids and proteins. Some cancers can become highly dependent on glutamine [1, 4, 9] such that the demand for glutamine outpaces supply. Additionally, some tumour cells *in vitro* are unable to survive in the absence of an exogenous supply of glutamine [8, 10]. Consequently cells develop a metabolic strategy to provide an alternative source of carbon other than glucose to derive carbon necessary to fuel the TCA cycle [11]. Recently, it has been shown that glutamine can enhance cancer progression independent of its metabolic role as it can act as a signalling agent to activate the transcription factor STAT3 which is required to mediate the proliferative effects of glutamine on cancer cells [12]. Furthermore, glutamine can indirectly activate other signalling pathways, such as the mammalian target of rapamycin complex 1 (mTORC1), a critical kinase that regulates cell growth and proliferation, as glutamine derived α -ketoglutarate is required for GTP loading of RagB and subsequent activation

of mTORC1[13]. Additionally, glutamine efflux through SLC7A5 is coupled by leucine uptake. The latter is known as a potent activator of mTORC1 [14].

The maintenance of redox homeostasis in cancer is important because the highly proliferating cells that encounter increased reactive oxygen species (ROS) production due to enhanced glutamine metabolism need a defence mechanism to avoid apoptosis [15]. Glutamine has a role in maintaining the redox balance through different mechanisms. Metabolites produced during the TCA cycle, serve as precursors for the reducing agent, Nicotinamide Adenine Dinucleotide Phosphate (NADPH). Furthermore, exchange of intracellular glutamate through the transporter SLC7A11 mediates the Cysteine uptake. This amino acid is then reduced to cysteine, the rate-limiting product for glutathione (GSH) biosynthesis [16]. Both, NADPH and GSH, act as a key regulator of cellular redox status [11, 15].

Glutaminase isoforms

In humans, glutaminase exists as two isoforms; kidney-type (GLS) and liver-type (GLS2), which not only differ in kinetic properties but also in protein structure and tissue distribution [17]. GLS and GLS2 are encoded by the *GLS* and *GLS2* genes respectively [18] and both can undergo alternative splicing to produce several variants (Figure 2). GLS (KGA; long transcript), GLS C (GAC; short transcript) and GAM are encoded by the *GLS* gene. However, GAM is significantly shorter than KGA or GAC and exhibits no measurable catalytic activity whereas GAC has greater catalytic activity and is frequently upregulated in cancer cells [19, 20]. GLS2 (LGA; shorter transcript) and GAB (long transcript isoform) splice variants encoded by the *GLS2* gene also exist [17, 18, 21].

Role of Glutaminase in Cancer

In cancer, the two GLS isozymes have opposing roles in tumourigenesis. GLS correlates with tumour growth rate and malignancy and is regulated by the oncogene c-MYC, whereas GLS2 tends to have tumour suppressive features and is regulated by p53 [17, 22-24]. Upregulation of GLS is observed in cancers including breast, liver, colorectal, brain, cervix, lung and melanoma [20, 25]. Rapidly growing malignant cells have elevated mRNA levels and en-

hanced GLS protein expression [24, 26-28] and GLS enzymatic activity correlates with poor disease outcome in liver, lung, colorectal, breast and brain tumours [24, 25, 29-32]. It is however the GAC variant which is a key enzyme for cancer cell growth [20, 28, 33, 34].

It appears that post-translational phosphorylation of GAC at specific regions of the enzyme by different signalling pathways can alter GLS activity [28, 33, 35]. Phosphorylation of GAC at Ser314 by oncogenic proteins Rho-C regulated PKC ϵ kinase is responsible for elevated GAC activity [35]. In contrast, serine 95 phosphorylation at the GLS N-terminal region leads to decreased GLS activity [33].

The expression of GLS2 variants are markedly increased in tumour cells that are more differentiated and is associated with a significantly prolonged survival time [24, 25, 36, 37]. GLS2 negatively regulates the activity of PI3K/AKT signalling [38] and Rac1 by mediating p53 function in HCC resulting in the inhibition of migration, invasion and metastasis of cancer cells [25, 27].

Glutamine Dependency in Breast Cancer

The need for glutamine varies according to different BC molecular subtypes [34-35, 41] where only some require an exogenous supply of glutamine and demonstrate glutamine dependence [38]. For example, triple-negative BC (TNBC) and HER2+ cell lines are highly glutamine dependent whereas luminal tumours have variable glutamine dependence [32, 39, 40]. Luminal A tumours are primarily glutamine independent as they exhibit only moderate effects on growth and viability in a glutamine-deprived environment whereas Luminal B cells show much higher glutamine metabolic activity [41].

Glutaminase in Breast Cancer

When interrogating Breast Cancer Gene-Expression Miner v4.3 (<http://bcgenex.centre-gauducheau.fr>), *GLS* and *GLS2* mRNA expression are negatively correlated (Figure 3). *GLS* is associated with high grade ($p=0.006$) whereas *GLS2* is associated with low grade ($p<0.0001$) tumours. In a relatively small study of breast tumours, high GLS protein was associated with high tumour grade and high grade metastatic BC but not tumour size, or nodal

stage [42][20]. In terms of BC patient outcome, *GLS* mRNA expression predicts poor patient survival (Figure 4a) and high *GLS2* mRNA predicts a better survival (Figure 4b).

In biological subtypes, *GLS* mRNA and/or protein expression is higher in basal-like/TNBC and HER2+ tumours and associated with poor-disease free survival in patients with positive lymph node metastasis [40]. Luminal B tumours have higher expression of GLS protein compared with Luminal A tumours and is predictive of poor patient outcome [30, 42-45] (Figure 3).

In contrast, *GLS2* mRNA is significantly higher in luminal A tumours compared with luminal B, HER2+ and TNBC (Figure 3). However, there is very little published information on GLS2 protein expression in cancer, including BC. Interestingly, patients with tumours expressing *GLS* but not *GLS2* confers a worse survival (Figure 4c) which is only observed in Luminal B tumours (Figure 4d).

Glutaminase Regulation in Cancer - Role of Oncogenes and Tumour Suppressor Genes

c-Myc plays a key role in the induction of glutamine dependence as it can enhance glutamine influx and metabolism. There is evidence that GLS, both splice variants KGA and GAC, are positively regulated by *c-myc* and are strongly expressed in c-Myc induced tumours [26, 34, 46, 47]. Tumours exhibiting overexpression of c-Myc with elevated GLS expression, together with a high influx of glutamine into the cells, consequently become glutamine addicted [10, 48].

c-Myc transcription stimulates GLS expression through different mechanisms [34, 48-50]. GLS is partly upregulated by microRNAs (miRNAs) where its translation is repressed by miR-23a/b through the mTORC1 pathway. Moreover, cancer cells that are dependent on Rho GTPase signaling via NF- κ B activity for progression of malignancy have activated GAC and consequently elevated level of GLS activity [28, 51, 52].

Conversely, GLS2 is induced by p53, in response to oxidative stress, to engage antioxidant responses in order to decrease ROS levels and participate in DNA damage repair processes [53]. Hu et al, 2010 showed that GLS2 is a p53 target gene containing p53 DNA-binding elements in the promoter region and that in turn GLS2 mediates p53 function in the regulation of energy metabolism and antioxidant defence in cells [22, 54].

The difference in the catalytic activity of GLS and GLS2 could be due to the underlying regulatory mechanisms. The deamination of glutamate by GLS results in the release of ammonia which is essential to support cell survival processes through providing alpha-ketoglutarate and intermediates for biosynthesis. However, glutamate produced from GLS2 activity supports the antioxidant machinery/mechanism (glutathione) in the cell cycle.

Potential Therapeutic Uses

Since glutaminase is critical for tumour growth and predominantly upregulated in highly proliferating breast tumours and a key enzyme in the first step of glutamine catabolism, it has the potential for acting as a target for therapy.

Glutaminase Inhibitors

6-diazo-5-oxo-L-norleucine (L-DON) GLS inhibitor is the earliest inhibitor of both GLS and GLS2 to be used in preclinical models. As a result of its non-selectivity and undesirable effects due to having similar structure to glutamine and having reactive chemical compounds, other compounds were developed [55]. Recently, two small molecules that inhibit both GLS and GLS2 have been determined: Bis-2-(5-phenylacetamido-1, 2, 4-thiadiazol-2-yl) ethyl sulfide (BPTES) and dibenzophenanthridine-968. *In vitro* and mouse model xenograft studies show that BPTES significantly inhibits GLS over GLS2 [28, 55, 56] in various cancer types. Whereas the 968-class of inhibitors inhibit GLS and GLS2 with similar potency [17, 57].

On the other hand, BPTES is a potent GLS inhibitor with minimal toxicity. The inhibitor does not exhibit structural similarities to glutamate or glutamine. However, it forms an inactive tetramer complex site where it interacts with GLS and not at the site where glutamine is catalysed. Thus there is no competition in the inhibition of GLS with the molecule [58].

BPTES inhibits GLS activity in glioma cells where glutamate and α -KG levels are decreased leading to a decrease in subsequent TCA cycle intermediates and its downstream products and slowed tumour growth [56]. BPTES also suppresses cell proliferation in HER2+ BC cells associated with increased GLS activity [59]. Although BPTES selectively inhibits GLS over GLS2 [53], it is described to have limitation in pharmacological application due to its poor metabolic stability, low solubility and moderate potency [57]. Like BPTES, Compound 968 is an allosteric inhibitor of GLS and inhibits the activity of KGA and GAC [60]. *In vitro* and mouse Xenograft model studies have shown anti-tumour activity of the compound in lymphoma, BC, ovarian and glioblastoma cells [24, 28, 61].

Very recently, evidence regarding inhibition of glutaminase as a therapeutic approach in treatment of cancer has resulted in the development of a BPTES derivative, 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy) phenyl) acetamido) pyridazin-3-yl) butyl)-1, 3, 4-thiadiazol-2-yl) acetamide (CB-839) [8]. Like BPTES, CB-839 is a member of the benzo[a]phenanthridinone family containing a pyridazine ring. The small molecule is a GLS inhibitor that regulates the enzymatic activity of KGA and predominantly the GAC splice variant isoenzyme [28, 40] by targeting the allosteric site of GLS. The inhibitor works by binding to and stabilising an inactive tetrameric state of the enzyme, rather than by competition with glutamine for binding to the active site where glutamine is hydrolysed [53, 56]. CB-839 is a more potent compound in terms of inhibition of GLS compared with BPTES [28, 55, 58]. In addition, CB-839 has an inhibitory concentration of 30- and 50-fold lower than BPTES [62].

Pre-clinical models demonstrate that CB-839 displays significant growth inhibition in certain subtypes of BC. Gross *et al*, 2014 demonstrated that TNBC are sensitive to CB-839 compared to luminal A/ER+ cells (MCF-7) mainly because of their high glutamine dependence and enhanced glutamine utilisation. Treatment of TNBC with CB-839 lowered levels of glutamate suggesting blockage of glutamine metabolism by inhibiting GLS [8]. Consistent with the findings, CB-839 inhibits signaling pathways in transformed cells via Rho GTPases which are linked to the activation of GLS hence, inhibiting the enzyme invasive activity. The treatment with CB-839 resulted in reduced TNBC growth in mice models injected with tu-

mour cells by half. However, the inhibitory effect of CB-839 on the growth of the other highly proliferative BC subtypes, i.e. luminal B and HER2+, has yet to be comprehensively confirmed.

FUTURE PERSPECTIVES

The GLS inhibitor CB-839 has already shown promising results in several solid cancers including TNBC and therefore has a strong therapeutic potential particularly in those exhibiting high glutamine dependency [63]. Phase I and II clinical trials currently being conducted are summarised in Table 2. A further GLS allosteric inhibitor, UPGL00004, shows similar potency in TNBC with additional growth inhibition in combination with the anti-vascular endothelial growth factor antibody bevacizumab [64].

Whilst TNBC have high dependence on glutamine, it certainly appears that glutaminase, particularly GLS but potentially GLS2, also plays an important role in the aggressive subclass of luminal BC. Therefore it is essential to elucidate the role of glutaminolysis in luminal B BC growth and progression and whether GLS offers a potential new therapeutic option for these BC patients whom have an uncertain prognosis due to relapse and/or development of resistance to current therapies.

Indeed, a most recent finding has provided some initial evidence that a luminal B-like patient xenograft is sensitive to CB-839 [65] demonstrating its potential use against other BC subtypes than TNBC alone. GLS inhibition in the luminal B xenograft model resulted in inhibition of the downstream metabolites proline and alanine indicating that its sensitivity is perhaps linked to it not being able to adapt to hypoxic environment through activation of proline mechanisms. It certainly suggests that BC subtypes might possibly be dependent on different glutamine metabolic characteristics.

Conclusion

Glutaminase plays a key role in various tumours including BC, which exhibit deregulated glutaminolysis because of overexpression and/or regulation of glutaminase. Both GLS isoen-

zymes are expressed in BC and GLS, particularly the GAC splice variant, is primarily linked to cancer progression and overexpression. Allosteric inhibitors, such as the small molecule CB-839, offers a unique opportunity to regulate this important metabolic enzyme. Clinical trials in TNBC and haematological malignancies are underway and look promising. However, there is still need to understand the role of both GLS and GLS2 in other rapidly proliferating BC subtypes including luminal B tumours where inhibition of BC could be a potential therapeutic approach, in addition to endocrine therapies that have limited success.

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Table 1. Glutaminase expression and clinical outcome in different cancers.

Cancer	Glutaminase expression	Observation on clinical outcome	References
Liver	GLS overexpressed	Poor prognosis	[25, 27, 67, 68]
	GLS2 overexpressed	Good prognosis	
Colorectal	GLS overexpressed	Poor prognosis	[29]
	GLS2 yet to be studied		
Lung	GLS overexpressed	Poor prognosis	[31, 58]
	GLS2 yet to be studied		
Leukaemia	GLS overexpressed	Poor prognosis	[69]
	GLS2 yet to be studied		
Lymphoma	GLS overexpressed	Poor prognosis	[40]
	GLS2 yet to be studied		
Breast	GLS overexpressed	Poor prognosis in TNBC & ER+ (highly proliferative)	[30, 32, 40, 42, 45]
	GLS2 varies among subtypes	Yet to be studied	
Melanoma	GLS overexpressed	Poor prognosis	[70]
	GLS2 yet to be studied		
Brain	GLS overexpressed	Poor prognosis	[24]
	GLS2 overexpressed	Good prognosis	
Prostate	GLS overexpressed	Poor prognosis	[40, 71]
	GLS2 yet to be studied		

Table 2. Clinical trials using GLS inhibitor CB-839

Cancer type	Clinical trial phase	Clinical Trial number
Renal Cell carcinoma	Phase II	NCT03428217
Melanoma	Phase I/II	NCT02771626
NSCLC	Phase I/II	NCT02771626
Colorectal	Phase I/II	NCT02861300
Myelodysplastic syndrome	Phase I/II	NCT02071927
Breast (TNBC)	Phase I/II	NCT02071862

Source: *National Cancer Institute*

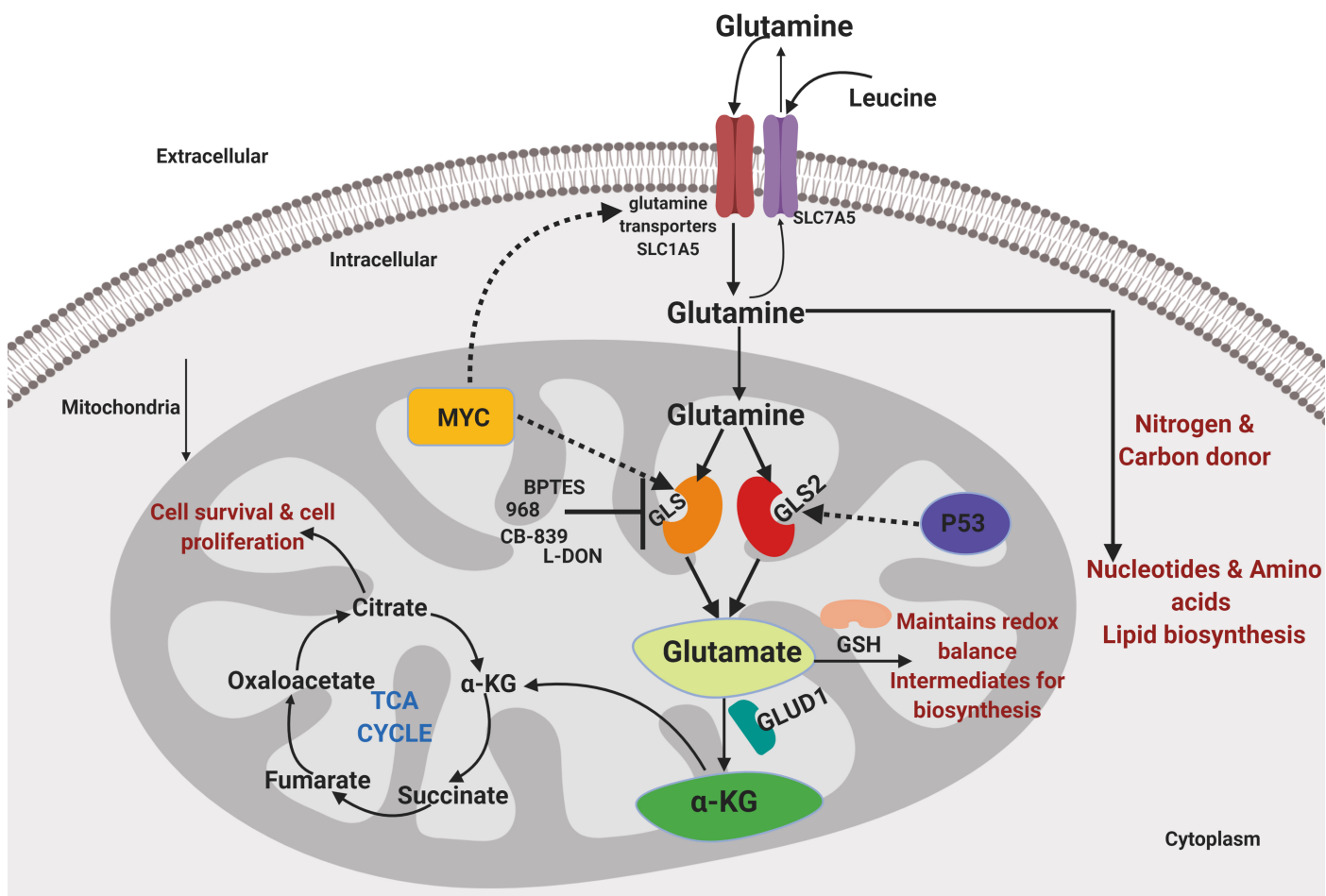
Figure 1: Glutamine metabolism in cancer cell. Glutamine is an essential amino acid that serves as a carbon and nitrogen source for energy production and nucleotide biosynthesis. Amino acid transporters regulate glutamine supply into the intercellular space. Glutamine is transported across the plasma membrane mainly by transporters: SLC1A5, SLC7A5. In the mitochondrion, either glutaminase, GLS, or GLS2 converts glutamine to glutamate. The latter is converted into α -ketoglutarate and enters the TCA cycle for processing. Oncogene c-MYC upregulation is responsible for the direct promotion of the expression of glutamine transporters enhancing glutamine entry into the cell and upregulation of GLS. Tumour suppressor p53 is a transcriptional target for the GLS2 gene increasing its expression under both stressed and non-stressed conditions. GLS inhibitors used to target GLS directly are 6-diazo-5-oxy-L-norleucine DON, 968, bis-2-(5-phenylacetamido-1, 2, 4-thiadiazol-2-yl) ethyl sulphide (BPTES) and CB-839 [1, 58, 66].

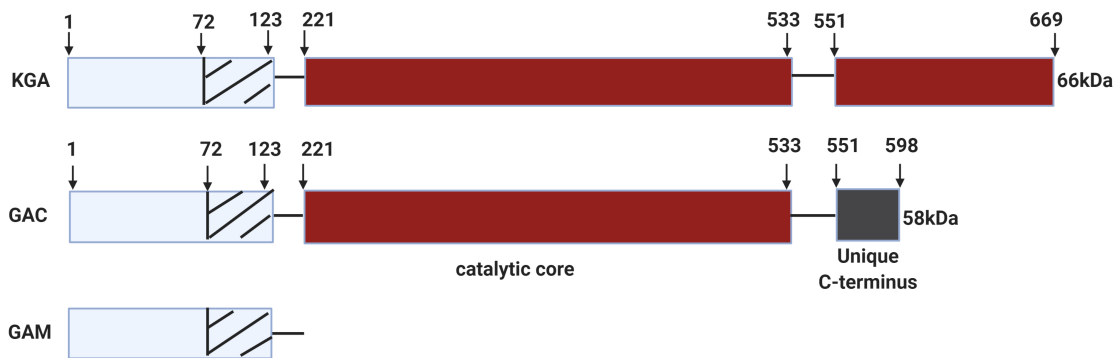
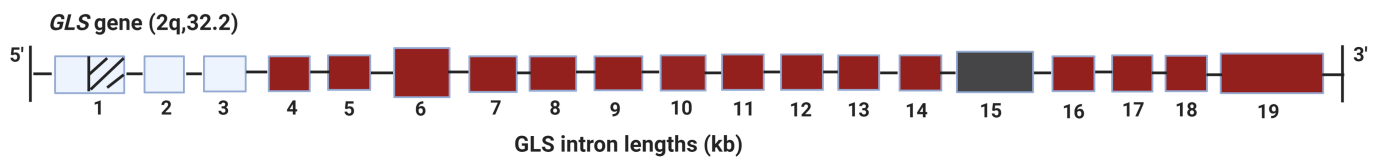
Figure 2: Structure of the *GLS* and *GLS2* gene. (A) *GLS* gene is 82kb and contains 19 exons on chromosome 2. Alternative splicing of the gene produce KGA and GAC splice variants, which share identical N-terminal region and GAM splice variant. KGA is derived from exon 1-14 and 16-19 while GAC protein is from 1-15 exons but have unique C-termini sequences. The central region is the catalytic active site. (B) *GLS2* has 18 exons that are 18kb on chromosome 12. Alternative splicing of the gene produce LGA (short transcript) and GAB (long transcript) isoforms. The splice variants share a common C-terminus and differ at their N-termini region. Adapted from [17, 67].

Figure 3: *GLS* and *GLS2* mRNA levels in the molecular subtypes of breast cancer and their correlation using GeneMiner (a, c, e) and METABRIC (b, d, f) datasets

Figure 4: Association of *GLS* and *GLS2* mRNA expression and patient outcome in breast cancer using GeneMiner for (a) *GLS* (b) *GLS2* showing low gene (less than the median) expression is associated with poor patient survival. Combined expression of *GLS* and *GLS2* mRNA expression and patient outcome in the METABRIC dataset for (c) all breast cancers (d) luminal B tumours.

Figure 5: Immunohistochemical expression of GLS and GLS2 protein in invasive ductal breast cancers of no special type. Cytoplasmic GLS showing homogenous and granular immunoreactivity, (a) x10 magnification and (b) x20, and GLS2, showing homogenous immunoreactivity, (d) x10 and (e) x20, expression. Negative controls for GLS (c, x10) and GLS2 (f, x10).





GLS2 gene (12q,13.3)



