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Nonessential amino acid metabolism in breast cancer

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ABSTRACT

Interest in studying cancer metabolism has risen in recent years, as it has become evident that the relationship between cancer and metabolic pathways could reveal novel biomarkers and therapeutic targets. Metabolic starvation therapy is particularly promising due to its low toxicity. Nonessential amino acids are promising metabolites for such therapy because they become essential in many tumor cells, including breast cancer cells. This review will focus on four nonessential amino acid metabolism pathways: glutamine–glutamate, serine–glycine, cysteine, and arginine–proline metabolism. Recent studies of these amino acids have revealed metabolic enzymes that have the potential to be effective as cancer therapy targets or biomarkers for response to metabolic starvation therapy. The review will also discuss features of nonessential amino acid metabolism that merit further investigation to determine their relevancy to breast cancer treatment.

Contents

1. Introduction

Breast cancer is the most frequently occurring cancer in women, and despite the development of new therapies there has been little decline in the mortality rate over the past decade (Siegel et al., 2015). This is partly due to the genetic diversity of breast cancers, such that there is no single therapy that is effective against all breast cancer subtypes. Breast cancers are commonly grouped by their hormone receptor status or specific mutations, in genes such as BRCA1/2 and PIK3CA, which can be used as biomarkers to predict the most efficacious targeted therapy for a breast cancer case (McCubrey et al., 2015, 2014). However, many breast cancers do not contain a single, targetable driver mutation, and targeted therapies are unlikely to

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eliminate all breast cancer cells due to tumor heterogeneity. Recently, new interest in studying the relationship between cancer and metabolism has arisen as a potential avenue to identify novel biomarkers and therapeutic targets effective across multiple breast cancer subtypes.

Cancer metabolism is not a new field; Otto Warburg's pioneering research first described the increased levels of aerobic glycolysis followed by lactate fermentation for energy production in cancer cells, since termed the "Warburg Effect" (Warburg, 1956). Since this discovery, glycolysis has been heavily explored in the context of cancer, resulting in the development of drugs that target glycolysis and oxidative phosphorylation, some of which are in clinical trials (Granchi et al., 2014; Kim, 2015; Ngo et al., 2015; Talekar et al., 2014). Other metabolic pathways have also been implicated in cancer, particularly folate metabolism, evidenced by the use of the antifolates methotrexate and pemetrexed as a frontline chemotherapies in multiple cancers, including breast cancer (Amelio et al., 2014). A potentially more efficacious strategy is metabolic starvation therapy, by removing or limiting the availability of a specific metabolite, as this has the potential to be less toxic to patients than chemotherapy or radiation (Changou et al., 2014). Nonessential amino acids are especially promising metabolites for starvation therapy. They can be synthesized by normal cells and thus an extracellular source is not required for their health, while many tumor cells require an external supply of nonessential amino acids (Morris, 2009; Souba, 1993). This difference can be utilized to selectively inhibit tumor cell growth by the removal of specific amino acids.

Amino acids may also serve as useful biomarkers for diagnosis as well as screening during treatment, since they can be easily measured in blood, saliva, and urine (Budczies et al., 2013; Kim et al., 2015). Recently, amino acid profiling of saliva from breast cancer patients identified fifteen amino acids with significantly changed levels between breast cancer patients and healthy controls, and which could serve as biomarkers for early detection and breast cancer diagnosis (Cheng et al., 2015).

This review summarizes four metabolic pathways for nonessential amino acids, focusing on their potential for applications as biomarkers and therapeutic targets in breast cancer treatment. The glutamine–glutamate and serine–glycine pathways have been well-studied in the past decade, whereas cysteine and arginine–proline metabolism have become the focus of more recent studies. The review concludes with an assessment of the field and understudied areas that may be valuable for the treatment of breast cancer.

2. Glutamine and glutamate metabolism

Of all the amino acids, glutamine is the most consumed by cancer cells (Jain et al., 2012). Glutamine is used for nucleotide and lipid biosynthesis, and also to synthesize glutamate, which can then be converted to alpha-ketoglutarate and feed the tricarboxylic acid (TCA) cycle (Fig. 1) (Budczies et al., 2013; Jeon et al., 2015). Glutaminolysis, the conversion of glutamine to glutamate for the production of energy via lactate, is increased in cancer cells (Erickson and Cerione, 2010). Specifically, glutamine can drive oxidative phosphorylation in cells transformed by Ras and Akt, mutations that occur in some breast cancers (Fan et al., 2013).

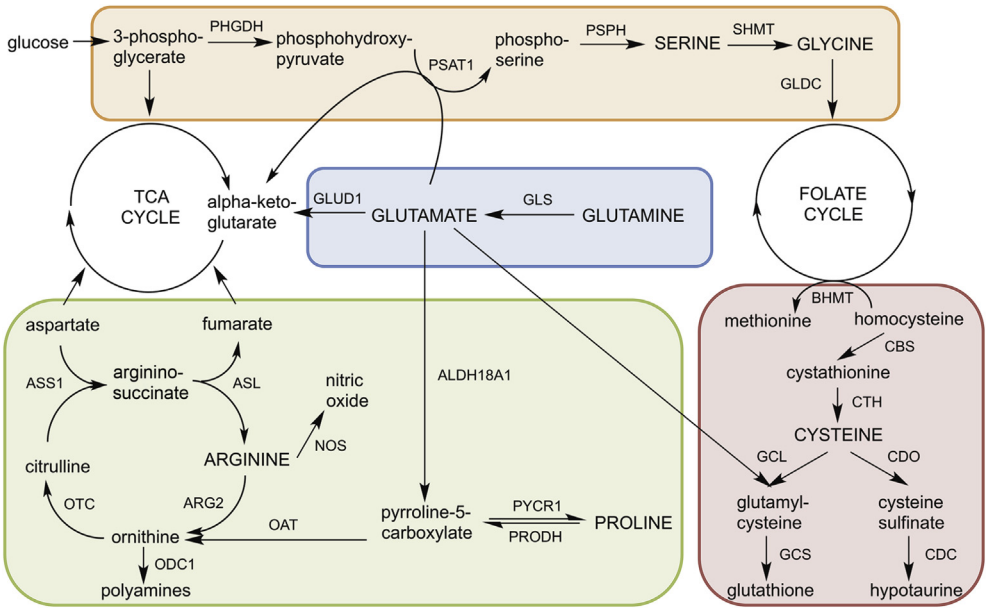

Fig. 1. Metabolic pathways of select nonessential amino acids. Nonessential amino acid pathways are interconnected and often feed the TCA cycle or enter one-carbon metabolism via the folate cycle, contributing to cancer cell growth. Serine–glycine metabolism in orange, glutamine–glutamate metabolism in blue, arginine–proline metabolism in green, and cysteine metabolism in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Increased glutamate levels in breast tumor versus normal breast tissue samples have been found to correlate with estrogen receptor (ER) status and endocrine resistance, indicating a change in regulation at the first step of glutaminolysis (Budczies et al., 2015, 2013; Cao et al., 2014; Shajahan-Haq et al., 2014). The observed patterns suggest that ER-negative breast cancer patients may benefit most from glutaminase (GLS) inhibition (Budczies et al., 2013). Among breast cancer subtypes, HER2-amplified breast cancers have the highest levels of glutamine metabolism, with increased GLS and glutamate dehydrogenase (GLUD1) expression (Cao et al., 2014; Kim et al., 2013), and triple-negative breast cancer (TNBC) exhibited increased expression of GLS and GLUL (van Geldermalsen et al., 2015), indicating that this molecular subtype of breast cancer may respond well to glutamine metabolic therapy. An increase in glutamine and glutamate also correlates with the expression of epithelial–mesenchymal transition (EMT)-associated transcription factors (Bhowmik et al., 2015), and thus has potential not only as a biomarker for choosing a metabolic therapy, but also for monitoring cancer progression and prognosis.

The specific mechanisms underlying glutamine dependency are still being explored, and current research indicates that the mechanisms likely vary between breast cancer subtypes. In *HER2*-positive breast cancer, increased levels of the transcriptional activator PGC-1α lead to expression of genes that modulate glutamine metabolism, including *GLS* and *GLUD1*, and expression of these genes correlates with poor prognosis. However, this pattern does not hold across other breast cancer subtypes, and therefore other mechanisms must also contribute (McGuirk et al., 2013). Glutamine metabolism also affects the response of cells to chemotherapy. In aromatase inhibitor (AI)-resistant breast cancer, crosstalk between *HER2* and ER upregulates glutamine metabolism via c-Myc. c-Myc elevation and glutamine metabolism increases the sensitivity of AI-resistant cells to inhibitors of GLS and the glutamine transporter SLC1A5, and to the removal of external glutamine (Chen et al., 2015; Shajahan-Haq et al., 2014). Glutamine may also play a role in response to paclitaxel. One mechanism by which paclitaxel promotes autophagy and cell death is inducing stress that leads to ubiquitin-mediated degradation of the glutamine transporters SLC1A5 and SLC38A2. This in turn decreases activity of the mechanistic target of rapamycin complex 1 (mTORC1), leading to increased autophagy. Inhibitors of SLC1A5 lead to similar effects in TNBC, thereby reducing glutamine uptake, mTORC1 signaling, and cell growth (van Geldermalsen et al., 2015). Moreover, SLC1A5/38A2 expression may also have promise as a subtype-specific biomarker, as its expression has been correlated with improved prognosis in luminal breast cancer (Jeon et al., 2015), while low expression correlates with increased survival of TNBC in xenograft experiments (van Geldermalsen et al., 2015).

While it has long been acknowledged that glutamine metabolism could be a therapeutic target, many early glutamine therapies presented significant toxicity or were ineffective *in vivo*. More recently, specific GLS inhibitors have been developed and are in clinical trials (Budczies et al., 2015). A study of metabotropic glutamate receptor expression in TNBC indicates that inhibition of receptor-decreased proliferation, and drugs such as Riluzole – already approved for treatment of amyotrophic lateral sclerosis – reduces tumor volume of mouse xenografts by up to 80% (Speyer et al., 2012). A greater understanding of the mechanisms by which glutamine and glutamate sustain cancer cells may lead to the development or repurposing of other successful targeted metabolic therapies and use of the pathway enzymes and metabolites as biomarkers.

3. Serine and glycine metabolism

Serine and glycine are synthesized from the glycolysis intermediate 3-phosphoglycerate by a series of enzymatic reactions (Fig. 1). The methyl groups of glycine can feed one-carbon metabolism in the folate cycle, which is utilized by cancer cells for the synthesis of proteins, nucleic acids, lipids, and cofactors (Amelio et al., 2014; Locasale, 2013). Serine and glycine are also involved in the synthesis of antioxidants, which increase the survival of cancer cells in hypoxic environments (Amelio et al., 2014). Glutamate is also a product of serine synthesis, and its conversion of alpha-ketoglutarate can feed energy production by the TCA cycle (Amelio et al., 2014).

Removal of serine and glycine leads to reduced proliferation of breast cancer cells, an effect that can be completely rescued by re-addition of serine but only partially by glycine. Many cancer cells preferentially take up serine over glycine, and when serine is lacking convert glycine into serine, consuming one-carbon units and depleting nucleotide pools (Labuschagne et al., 2014). This indicates a specific role for serine in supporting breast cancer proliferation by a mechanism other than one-carbon metabolism, and this remains to be explored. Additionally, certain breast cancer cell lines exhibit robust growth inhibition in the absence of serine, indicating that serine starvation may be a successful metabolic therapy in specific cases (Labuschagne et al., 2014). Classification of the differences between breast cancer cells that exhibit decreased proliferation in comparison to those that cannot proliferate at all in the absence of serine could reveal potential biomarkers for response to serine deprivation as a therapeutic approach.

Several enzymes in serine and glycine metabolism have been proposed as biomarkers for cancer stage and prognosis. Phosphoglycerate dehydrogenase (PHGDH), phosphoserine phosphatase (PSPH), and serine hydroxymethyltransferase (SHMT) levels are high in TNBC and low in luminal-A breast cancers (Kim et al., 2014). Breast cancer patients whose tumors express high levels of mitochondrial glycine pathway components have worse prognosis than those with low expression levels (Jain et al., 2012), specifically expression of PHGDH and SHMT (Noh et al., 2014). Across multiple subtypes, high PHGDH and PSPH with low SHMT correlates with short overall survival, and high PSPH and low glycine dehydrogenase (GLDC) with short progression-free survival (Kim et al., 2014). From these and other studies, PHGDH, phosphoserine aminotransferase (PSAT), PSPH, and SHMT may all be useful biomarkers for determining breast cancer prognosis (Antonov et al., 2014; Kim et al., 2014).

Copy number increases of the *PHGDH* gene lead to a 70% increase in PHGDH protein levels in ER-negative breast cancer, moving products of glucose from glycolysis to production of serine and glycine (Possemato et al., 2011). These elevated levels of PHGDH most closely correlate with TNBC and basal-like breast cancer (Locasale et al., 2011; Noh et al., 2014). Suppression of PDGDH expression decreases cell proliferation by lowering production of alpha-ketoglutarate, as serine contributes up to 50% of glutamate flux into the TCA cycle in PHGDH-overexpressing cells (Possemato et al., 2011). Overexpression of PHGDH in MCF-10A breast cells also leads to increased proliferation and anchorage-independent growth, indicating a critical role in transformation (Locasale et al., 2011). Coupled with the knowledge that non-tumorigenic epithelial cells do not require PHGDH for growth (Locasale et al., 2011), suggests that PHGDH is an attractive therapeutic target and biomarker (Amelio et al., 2014). PHGDH contains many binding sites for which specific inhibitors could be designed, and nonspecific inhibitors are already available (Locasale and Cantley, 2011). However, PHGDH deficiencies in children lead to neurological defects, so agents would need to be developed with limited access to the central nervous system, and with therapeutic windows carefully calculated (DeBerardinis, 2011).

Serine and glycine metabolism contains many nodes of regulation that can be modulated to decrease the proliferation of breast cancer cells. Especially promising are limiting the intake of serine to deplete nucleotide pools and slow proliferation, and targeting PHGDH and SHMT (Amelio et al., 2014). PHGDH also has potential value as a biomarker for breast cancer staging and prognosis, as well as for predicting response to targeted serine metabolism therapies (Amelio et al., 2014).

4. Cysteine metabolism

It has long been known that many tumor cells, including some breast cancer cell lines, cannot survive when methionine is replaced by homocysteine, while non-malignant cells survive. Targeting methionine metabolism to combat cancer is has been explored in some detail, and is reviewed elsewhere (Hoffman, 2015). However, prolonged methionine restriction can result in toxic side effects, so treatment options other than dietary restriction and methioninase treatment have gained attention (Cellarier et al., 2003). One such option is targeting cysteine metabolism, as it is involved in methionine metabolism (Fig. 1) but unlike methionine, cysteine itself is not an essential amino acid.

Alterations in cysteine levels have been observed in breast cancer, but have not been studied as extensively as glutamine or serine for their potential as biomarkers for different subtypes and staging of breast cancer. Implantation of human breast cancer cells into mice leads to an increase in plasma cysteine and homocysteine as the tumor progresses, indicating that cysteine may have potential as a marker for disease progression (Al-Awadi et al., 2008). Specifically, in *PIK3CA*-mutant breast cancer cells, cysteine levels increase by 13%. Coupled with the concomitant increase in glutamine, this could account for the observed increase in glutathione levels and allow cells to survive in the presence of elevated levels of reactive oxygen species (ROS) (Kim et al., 2015).

Modulating the ability of tumor cells to detoxify ROS is a key mechanism by which cysteine metabolism affects tumor cell survival. One study observed hypermethylation of cysteine dioxygenase (*CDO1*) in 60% of tested breast cancer samples, leading to increased ROS detoxification and tumor cell survival. Levels of methylation increase with tumor stage, and correlate with poor prognosis since they are not observed in normal cells. Restoring functional levels of CDO1 decreases tumor cell growth and sensitizes cells to doxorubicin (Jeschke et al., 2013). Cysteine also appears to be involved in resistance to doxycycline and tamoxifen, thereby affecting antioxidant production. Doxycycline-resistant MCF-7 cells have elevated levels of cystathione-beta-synthase (CBS), methylenetetrahydrofolate reductase (MTHFR), and betaine-homocysteine S-methyltransferase (BHMT), leading to increased transsulfuration of methionine and homocysteine to cysteine and decreased glutathione synthesis, rendering doxycycline-resistant cells more sensitive to oxidative stress (Ryu et al., 2011). By contrast, tamoxifen-resistant MCF-7 cells exhibit increased usage of homocysteine for cysteine production, but this leads to increased production of glutathione and subsequent decreased sensitivity to oxidative stress. Tamoxifen-resistant cells are sensitive to inhibition of cysteine metabolism, whereas non-resistant MCF-7 cells remain viable (Ryu et al., 2013). Therefore, cysteine metabolism may be a potential target to re-sensitize breast cancer cells to therapies such doxycycline and tamoxifen, but a greater understanding of the mechanistic connections to cysteine metabolism and the opposing effects on antioxidant production is clearly required. While these studies indicate that cysteine metabolism is a promising target for breast cancer therapy, additional work is required to determine its roles in multiple molecular subtypes of breast cancer and its potential as a biomarker. The ability to affect the antioxidant capabilities of cancer cells by targeting cysteine metabolism is an obviously attractive proposition however, since modulation of cysteine is likely to be better tolerated by normal cells than is methionine restriction.

5. Arginine and proline metabolism

Arginine and proline are nonessential amino acids in humans, although arginine can become conditionally essential in pathophysiology (Morris, 2009). Arginine and proline are generated from glutamate, with arginine as a key member of the urea cycle (Fig. 1). Arginine metabolism intersects with many other metabolic pathways, including nitric oxide, creatine, urea, and polyamine metabolism (Fig. 1), which can promote tumor growth (Morris, 2009). Proline can also feed the TCA cycle through the urea cycle, and its oxidation by proline dehydrogenase (PRODH) leads to the formation of ROS (Phang et al., 2015).

Increased levels of free arginine have been observed in many malignant breast tumors compared to benign tissues (Park et al., 1991), and many breast cancer patients have decreased plasma arginine (Vissers et al., 2005). Both observations suggest

potential applications as biomarkers for cancer diagnosis and prognosis, though the mechanisms leading to these changes are presently unknown. Arginine can become conditionally essential in some tumors. Classic examples are arginine auxotrophic tumors such as malignant melanoma, hepatocellular carcinoma, prostate cancer, and acute lymphoblastic leukemia, which cannot synthesize arginine due to a lack of argininosuccinate synthase (ASS) (Delage et al., 2010; Ensor et al., 2002). Because of this, arginine depletion via conversion to citrulline by ADI-PEG20 is an effective treatment for these cancers (Qiu et al., 2015), though citrulline itself is a highly effective precursor of arginine in cells expressing ASS and arginosuccinate lyase (ASL) (Kaore et al., 2013). Certain breast cancer cell lines are arginine auxotrophs due to low levels of ASS, including MDA-MB-231 and ZR-75-1 (Qiu et al., 2014), and in a study of 55 breast tumor specimens, 5 were reported to not express ASS (Dillon et al., 2004). These arginine auxotrophs do respond to arginine deprivation via ADI-PEG20, which induces mitochondrial oxidative stress and autophagy (Qiu et al., 2014). While breast cancers that express higher levels of ASS do not respond to ADI-PEG20 alone (Ensor et al., 2002), sensitization by this compound to ionizing radiation via downregulation of c-Myc and upregulation of p21 has been reported (Park et al., 2008). This may therefore be a more general combination therapy strategy for breast cancer patients.

Due to metabolic variations, it is important to consider arginine metabolism and dependency in specific contexts to identify precise patterns. This is best illustrated for ASS: while ASS deficiency correlates with worse prognosis in sarcomas, ASS levels positively correlate with a poor prognosis in gastric cancer and some breast cancers (Huang et al., 2013; Qiu et al., 2015; Shan et al., 2015). Effects on cell migration also vary, as ASS overexpression in myxofibrosarcoma inhibits migration (Huang et al., 2013), but knockdown or arginine withdrawal from gastric cancer cells inhibits cell migration (Huang et al., 2013; Shan et al., 2015). The reasons for these opposing observations is not known, nor is it clear if there are underlying expression patterns that can predict response to ASS and arginine deprivation, so this is an area of work that clearly requires additional investigation.

It is also important to note that even within breast cancer, distinct cell lines do not display the same response to arginine metabolic therapy. One study observed that MCF-7 and MCF-10A cells became quiescent upon arginine removal, but upon arginine re-addition MCF-7 recovered while MCF-10A senesced and did not recover (Chiaviello et al., 2012). A separate study noted the same effects in MCF-7 cells, whereas ZR-75-1 cells underwent cell death in the absence of arginine (Scott et al., 2000). While these are intriguing results, the mechanistic basis for these observations are not fully elucidated, although one study concluded that one determinant is expression levels of ASS, or polyamine synthesis, while another study of seven breast cancer lines implicated arginase (ARG) and nitric oxide synthase (NOS) expression (Singh et al., 2000). A clear pattern across multiple breast cell lines has yet to emerge, indicating a need to identify biomarkers that can predict tumor response to arginine metabolism therapy before such therapies can become clinically relevant.

Some therapeutic options are available to deplete arginine, but have not yet been applied to breast cancer (Qiu et al., 2015). ADI-PEG20 and rhArg-PEG both metabolize arginine, but require a deficiency in at least one urea cycle enzyme to prevent the re-synthesis of arginine (Ensor et al., 2002; Qiu et al., 2015). One study found that the arginase/ornithine decarboxylase inhibitor N-omega-hydroxy-L-arginine (NOHA) increases apoptosis of breast cancer cells that overexpress ARG (Singh et al., 2001, 2000), so inhibitors of arginine metabolism as well as arginine depletion strategies have promise for breast cancer therapy. Pyrroline-5-carboxylate reductase (PYCR1) has also been identified as a potential therapeutic target, as its depletion reduces tumor forming ability, indicating that some breast cancers may be highly dependent on proline synthesis (Possemato et al., 2011). However, as exhibited by the highly variable responses to ADI-PEG20 based on ASS status (Qiu et al., 2014), it is important to identify biomarkers to accompany each of these proposed therapeutic strategies in order to apply treatment that will elicit the most robust response.

6. Concluding remarks

Targeting nonessential amino acid metabolism is an emerging field for cancer therapy, especially in breast cancer, as it shows promise to selectively target aspects of tumor metabolism with minimal toxic side effects. Various metabolites and metabolic enzymes have merit as biomarkers and therapy targets for monotherapies or combination therapies. Moreover, amino acid metabolite levels in plasma have potential as accessible biomarkers for determining treatment regimens, and to follow during treatment to determine outcome and therapy response. The expression levels of proteins that modulate amino acid metabolism may also be useful biomarkers for selecting appropriate metabolic therapies for precision-guided medicine.

It is also worth noting that while glutamine and serine metabolism have been well studied in the context of breast cancer, the metabolism of other nonessential amino acids is a more nascent field. In a recent study that profiled metabolites in breast tumors, alanine was the most significantly altered metabolite, with especially high levels in ER-negative breast cancers and a two-fold increase in tumor over normal. This most closely correlated with downregulation of 4-aminobutyrate aminotransferase (ABAT), which converts alanine to malonate semialdehyde to feed the TCA cycle. Low ABAT expression is associated with poor prognosis, so high levels of alanine may contribute to aggressive breast cancer (Budczies et al., 2013). This illustrates the need for significant future work to determine the merit of targeting alanine metabolism for breast cancer therapy, and also indicates that potential new clinical targets can be identified through investigating metabolic pathways in cancer.

In conclusion, we posit that nonessential amino acid metabolism provides potential application for dietary restriction or targeted therapies, as well as the ability to enhance the effects of existing chemotherapies and also to re-sensitize drug-

resistant tumors. As interest in studying cancer metabolism grows, new therapeutic targets and biomarkers can be identified and further developed to provide new treatment modalities for breast cancer patients.

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