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Review Article

AMP-activated protein kinase and energy balance in breast cancer

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Abstract: Cancer growth and metastasis depends on the availability of energy. Energy-sensing systems are critical in maintaining a balance between the energy supply and utilization of energy for tumor growth. A central regulator in this process is AMP-activated protein kinase (AMPK). In times of energy deficit, AMPK is allosterically modified by the binding of increased levels of AMP and ADP, making it a target of specific AMPK kinases (AMPKKs). AMPK signaling prompts cells to produce energy at the expense of growth and motility, opposing the actions of insulin and growth factors. Increasing AMPK activity may thus prevent the proliferation and metastasis of tumor cells. Activated AMPK also suppresses aromatase, which lowers estrogen formation and prevents breast cancer growth. Biguanides can be used to activate AMPK, but AMPK activity is modified by many different interacting factors; understanding these factors is important in order to control the abnormal growth processes that lead to breast cancer neoplasia. Fatty acids, estrogens, androgens, adipokines, and another energy sensor, sirtuin-1, alter the phosphorylation and activation of AMPK. Isoforms of AMPK differ among tissues and may serve specific functions. Targeting AMPK regulatory processes at points other than the upstream AMPKKs may provide additional approaches for prevention of breast cancer neoplasia, growth, and metastasis.

Keywords: Breast cancer, AMP-dependent protein kinase, biguanides

Much attention is currently being paid to altered metabolism as it relates to progression of breast cancer [1]. Observations of responses to metformin in diabetic patients has been an instigator of this approach [2, 3]. As one of the primary points in the triage of energy flow, AMP-activated protein kinase (AMPK) is the focus of attention. AMPK responds allosterically to increases in the ratio of AMP/ATP, enhancing AMPK activation by AMPK kinases (AMPKKs) [4]. AMPK is also modified indirectly by hormones, adipokines, sirtuin-1, and metabolic products. The effect of AMPK activation on aromatase within the breast is of particular interest in postmenopausal women with breast cancer [5]. Synergism of biguanides with tamoxifen, aromatase inhibitors or trastuzumab/lapatinib may have particular application in breast cancer. This review is focused on the regulation of metabolism by AMPK in breast cancer and the optimal application of biguanides for treatment.

Structure of AMPK

AMPK plays an important role in energy balance at both cellular and whole body levels by balancing nutrient supply and demand. It is a heterotrimeric complex that consists of a catalytic (α) and two regulatory (β and γ) subunits [4, 6, 7]. There are two isoforms of the AMPKα subunit (AMPKα1 and AMPKα2), two isoforms of the β subunit (AMPKβ1 and AMPKβ2), and three isoforms of the γ subunit (AMPKγ1, AMPKγ2, and AMPKγ3), each of which is encoded by a separate gene [6]. The catalytic α-subunit is composed of a serine/threonine kinase domain containing a threonine residue (Thr172) that is phosphorylated by upstream kinases, an autoinhibitory domain (AID) with a negative effect on kinase activity, a linker region, and an α-subunit carboxy-terminal domain (α-CTD). A flexible serine-threonine-rich loop (ST loop) within the α-CTD can be phosphorylated by Akt. The AMPKβ subunit contains a carbohydrate-bind-

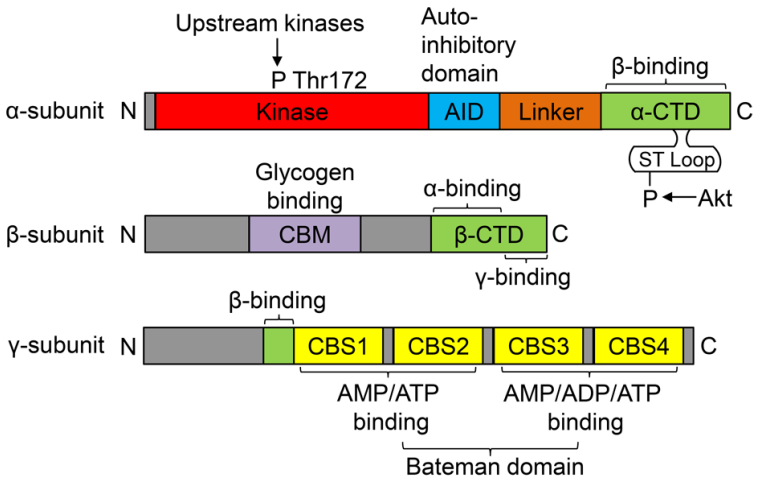

Figure 1. Domain structure of typical mammalian AMPK. AMPK complexes are heterotrimers composed of α-, β- and γ-subunits in a 1:1:1 ratio. The β-subunit carboxy-terminal domain (β-CTD) forms the core of the AMPK complex, binding to the α-CTD sequence in the α-subunit and the β-binding sequence in the amino terminus of the γ-subunit (green). The β-subunit also contains a carbohydrate-binding module (CBM) that is a binding site for glycogen (purple). The catalytic α-subunit contains conventional serine/threonine kinase domains with a threonine residue (Thr172) that is phosphorylated by upstream kinases (red). The kinase domain is followed by an auto-inhibitory domain (AID; blue) that has a negative effect on kinase activity. The AID is connected to the α-CTD by a less well conserved linker (orange). A flexible serine-threonine-rich loop (ST loop) within the α-CTD can be phosphorylated by Akt. The γ-subunit contains variable NH2-terminal regions followed by the short β-subunit binding sequence, then four tandem repeats of cystathionine β-synthase (CBS) motifs that act in pairs to form the binding sites for adenine nucleotides in mammalian AMPK (yellow). There is one binding site between CBS1 and CBS2 and two binding sites between CBS3 and CBS4. Four CBS domains are paired to form two Bateman domains.

ing module (CBM) that binds glycogen and a β-CTD region that serves as a bridge between the α- and γ-subunits. The AMPKγ subunit contains variable NH2-terminal regions followed by a short sequence involved in β-subunit binding, then four cystathionine β-synthase (CBS) domains that are paired to form two Bateman domains. These domains are the sites to which phosphorylated adenosines (AMP and ADP) bind to activate AMPK signaling [4] (**Figure 1**).

It is likely that all cells in the body express AMPK. The AMPKα1 isoform is widely distributed among organ systems, whereas the AMPKα2 isoform is limited to muscle, liver, and adipose tissue [8]. The heart, skeletal muscle, and liver are the most studied organs in terms of AMPK activity, but brain and adipose tissue have also been studied intensively. Human skeletal muscle expresses primarily the α2, β2, and γ3 isoforms [9]; however, after prolonged exercise, the AMPKα1 isoform is specifically increased [10]. Higher levels of AMPK are expressed in subcutaneous adipose tissue than in visceral fat [11], and subcutaneous but not visceral adipose tissue is negatively correlated with serum adiponectin levels in obese individuals [12].

Activation of AMPK

Activation of AMPK is accomplished by competitive replacement of ATP on the AMPKγ subunit by ADP or AMP (**Figure 1**). Saturation of the AMP binding site on the AMPKγ subunit leads to a thousand-fold activation of AMPK by upstream AMPKKs [13]. Although ADP has significantly lower affinity for AMPK, its much greater concentration in stressed cells makes it an important regulator of AMPK [7]. Binding of the first AMP or ADP to the CBS domain in the AMPKγ subunit triggers a conformational change, opens the heterotrimeric complex, and exposes the catalytic domain of the AMPKα subunit to AMPKK phosphorylation on Thr172. AMP or ADP binding to AMPK also attenuates the deactivation of AMPKα by protein phosphatase 2Cα and increases the duration of Thr172 phosphorylation of the AMPKα subunit [13]. Binding of the first AMP or ADP additionally increases the affinity of the binding site in the CBS domain of the AMPKγ subunit for a second phosphorylated adenosine (AMP or ADP) [14]. Three isoforms of the AMPKγ subunit have been identified, and the binding capacity of AMP and ADP differ among them [15]. The activation of AMPK leads to shift towards energy production and storage (anabolism) and away from energy utilization (catabolism) and cell growth and proliferation.

Activation of AMPK requires phosphorylation of the Thr172 in the AMPKα subunit by calcium-calmodulin protein kinase kinases (CaMKKs) and/or another AMPKK consisting of LKB1 (*STK11*), a scaffolding protein called mouse protein 25, and the STE-related adapter [16-

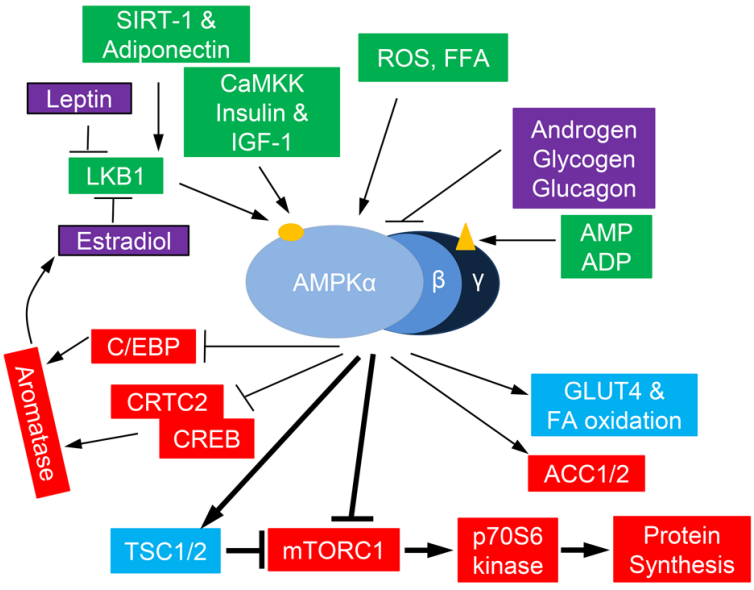

Figure 2. Activation of AMPK by endogenous hormonal and metabolic factors. Pathways that activate AMPK are shown in green and pathways that inhibit AMPK are shown in purple. Within the AMPK heterotrimer, the AMP/ADP binding site(s) are shown on the γ-subunit (triangle); the phosphorylation site (Thr172) is shown on the α-subunit (oval). Downstream targets of AMPK are shown in blue and red, leading to stimulation of energy storing pathways (blue) and inhibition of energy utilization pathways (red), as well as inhibition of aromatase.

18]. Formation of this LKB1 complex is facilitated by deacetylation of LKB1 via sirtuin-1 and, thus, there is an interdependency between the distinct energy sensing regulators AMPK and sirtuin-1 [16, 19]. The LKB1 complex phosphorylates at least 13 different AMPK-related protein kinases, although none of the AMPK-related kinases are activated by AMPK-activating metformin or AMP analogs [20]. In addition, Oakhill et al. [14] have shown that myristoylation of the AMPKβ subunit is required for activation of AMPK. Loss of β-subunit myristoylation abolishes AMPK activation by AMP. In the human (but not in the mouse), stress upregulates transcription of the β1- and β2-subunits of AMPK via a p53-dependent process, increasing the activity of AMPK in skeletal muscle, heart, white fat, and liver [21]. Alternatively, oxidative stress may activate AMPK by increasing cellular AMP and ADP [22]. Many other factors have significant allosteric effects on activity of AMPK, as reviewed by Hardie et al. [4].

Interactions of AMPK with endogenous hormonal and metabolic factors

AMPK is recognized as a master sensor of energy homeostasis and is a hub for the convergence of endocrine signals, including estradiol, androgens, inflammatory factors, leptin, and adiponectin, that modulate the energy balance [23, 24] (**Figure 2**, **Table 3**).

Sex steroid hormones

Estrogens play a major role in the modulation of energy balance through central and peripheral actions. Estradiol inhibits AMPK through estrogen receptor α (ERα) in the ventromedial nucleus of the hypothalamus, leading to stimulation of thermogenesis in brown adipose tissue and weight loss through activation of the sympathetic nervous system [25]. On the other hand, both estrogen [26, 27] and leptin [28, 29] inhibit AMPK phosphorylation in the liver and adipose tissue by inhibiting LKB1 activity. Androgens have a similar effect in adipocytes. Both testosterone and dihydrotestosterone (DHT) inhibit AMPK activation [30] and increase food intake [31].

Insulin, growth factors, and adipokines

Insulin- and insulin-like growth factor-1-induced Akt phosphorylation suppresses phosphorylation of the AMPKα subunit at Thr172, decreasing activation of AMPK and increasing energy utilization [32, 33]. While leptin, produced by adipose tissue, has inhibitory effects in the liver and muscle, adiponectin has stimulatory effects in both liver and muscle [34]. Exercise [33] and adiponectin [35] increase AMPK activation in both adipose tissue and muscle. In obesity, the ratio of leptin to adiponectin in adipose tissue is elevated, with a net effect of decreasing AMPK activation; however, this effect can be partially overcome by treatment with biguanides such as metformin [36]. Adiponectin activation of AMPK is also described in studies of various breast cancer cell lines. Treatment of MCF-7, T47D, and MDA-MB-231 breast cancer cells with adiponectin results in increased cytoplasmic LKB1 accumulation. This increases the activity of AMPK by stimulating of AMPKα phosphorylation at Thr172 without changing total

Table 1. Phosphorylation of AMPK and the distribution of AMPK subunits in normal, benign, and malignant breast tissue

AMPK \ Tissue	Normal Breast Tissue	Benign Breast Tissue		Malignant Breast Tissue
		Hyperplastic	Fibroadenoma	
p-AMPK	Present	Present	Present	Decreased
α-subunit	α1≥α2	α1, α2	α1, α2	α1 no change, α2 decreased
β-subunit	Unknown	Unknown	Unknown	Unknown
γ-subunit	Unknown	Unknown	Unknown	Unknown

p-AMPK, phosphorylated AMPK.

Table 2. The effect of AMPK-activating biguanides on different types of breast cancers

Effect of biguanides \ Breast cancer types		ER positive	HER positive	Triple negative
Systemic effect	(indirect effect, decreased serum glucose, insulin and IGF-I levels)	Yes	Yes	Yes
Direct effect	Inhibited breast mTOR function	Yes	Yes	Yes
	Decreased breast estrogen levels	Yes	?	?

AMPK protein expression levels [37]. Differential effects of adiponectin in breast tumors may be dependent on the presence or absence of ERα. Adiponectin promotes growth of ERα-positive ($ERα^+$) MCF-7 breast tumor cells and inhibits growth of ERα-negative ($ERα^-$) MDA-MB-231 breast tumor cell xenografts [38]. AMPK is necessary for the action of adiponectin on suppression of cyclin D1 and cell cycle arrest in both HepG2 (liver cancer cells) and MCF-7 cells [39]. FoxO3a is an intermediate in this process.

Metabolic intermediates

Essential fatty acids, except for oleic acid [40] and 3-phosphoglycerate [16, 41], increase the activity of AMPK. Glycogen has been shown to suppress the activation of AMPK by binding to the specific CBM on the AMPKβ subunit [28, 42] and glucagon inhibits AMPK via increased PKA-mediated inhibitory phosphorylation of serine 173 and reduced phosphorylation at Thr172 on the AMPKα subunit [43].

Other factors

Accumulation of NAD or NADH in cells is not a stimulus for activation of AMPK [13]. Based on nutrient availability, the other main sensor of energy balance, sirtuin-1, a histone deacetylase, is stimulated by NAD and inhibited by NADH and nicotinamide to increase energy availability [16]. In addition, the relationship between energy needs and activation of AMPK is not strict. For example, AMPK activation in response to exercise was suppressed in a study of trained subjects compared with sedentary individuals [10]. Exercise training markedly increased LKB1 and MO25 but did not increase AMPK [44]. Caloric restriction in human subjects had very little effect on AMPKα1 or AMPKα2 activities in heart or skeletal muscle, whereas plasma leptin increased by 8-fold [45].

Exogenous AMPK activators and inhibitors

Biguanides

The biguanides (metformin and phenformin) activate the AMPK pathway, inhibit protein and lipid synthesis, and elicit anti-tumor effects in breast tissue (**Table 3**). The biguanides act, in part, through facilitating LKB1 phosphorylation of AMPKα at Thr172 [46, 47]. Metformin and phenformin also activate AMPK by inhibiting complex 1 of the mitochondrial respiratory chain, causing a decrease in formation of ATP and an increase in AMP accumulation [42, 48]. Metformin is the most commonly used anti-diabetic drug and has been associated with decreased risk of breast cancer [49], probably due, at least in part, to activation of the AMPK pathway in the liver, which decreases circulating levels of insulin and glucose [50, 51]. Although phenformin has been withdrawn by the FDA for treatment of diabetes because of its major side effect of lactic acidosis, it may have an advantage over metformin in breast cancer treatment and prevention. First, phen-

Table 3. Recent publications on AMPK

Interaction of AMPK with endogenous hormonal and metabolic factors
Toyama et al., 2016: AMP-activated protein kinase mediates mitochondrial fission in response to energy stress [94]. Activation of AMPK promotes mitochondrial fragmentation through an identified mitochondrial fission factor.
Shrestha et al., 2016: Critical role of AMPK/Fox03 in globular adiponectin-induced cell cycle arrest and apoptosis in cancer cells [39]. Suppression of AMPK blocked adiponectin-induced cell cycle arrest in both HepG2 and MCF-7 cells.
Mauro et al., 2015: Estrogen receptor-alpha drives adiponectin effects on cyclin D1 expression in breast cancer cells [38].
Guo, et al., 2015: Synergistic antitumor activity of vitamin D3 combined with metformin in human breast carcinoma MDA-MB-231 cells involves m-TOR related signaling pathways [62].
Exogenous AMPK activators and inhibitors
de Queiroz et al., 2015: Metformin reduces the Walker-256 tumor development in obese-MSG rats via AMPK and FOXO3a [36].
Liu et al., 2015: Phenformin induces cell cycle change, apoptosis, and mesenchymal-epithelial transition and regulates the AMPK/mTOR/p70s6k and MAPK/ERK pathways in breast cancer cells [88].
Fan et al., 2015: Metformin exerts anticancer effects through the inhibition of the Sonic hedgehog signaling pathway in breast cancer [90].
Rice et al., 2015: Dual effect of metformin on growth inhibition and oestradiol production in breast cancer cells [57].
Expression of AMPK subunits in tumor tissue
Ross et al., 2016: Differential regulation of AMP and ADP of AMPK complexes containing different gamma subunit isoforms [15]. Activities of AMP, ADP, CaMKKβ, and A769662 on AMPK isoforms.
Mechanism of AMPK activity in the proliferation, apoptosis, and migration of breast cancer cell lines
Gollavilli et al., 2015: AMPK inhibits MTDH expression via GSK3beta and SIRT1 activation: potential role in triple negative breast cancer cell proliferation [82]. Metformin downregulated the oncogenic protein metadherin.
Tanaka et al., 2015: Mild glucose starvation induces KDM2A-mediated H3K36me2 demethylation through AMPK to reduce rRNA transcription and cell proliferation [83].
Jhaveri et al., 2015: AMP-activated kinase (AMPK) regulates activity of HER2 and EGFR in breast cancer [89]. The growth factors have sequences that are potential substrates for AMPK.
Domenech et al., 2015: AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest [99].
Li et al., 2015: p53 is required for metformin-induced growth inhibition, senescence and apoptosis in breast cancer cells [93].
Chou et al., 2014: AMPK reverses the mesenchymal phenotype of cancer cells by targeting the Akt-MDM2-Foxo3a signaling axis [81].
AMPK and human breast cancer
Zadra et al., 2015: Dissecting the dual role of AMPK in cancer: From experimental to human studies [1].
Hadad et al., 2015: Evidence for biological effects of metformin in operable breast cancer: biomarker analysis in a pre-operative window of opportunity randomized trial [113].
Dowling et al., 2015: Changes in insulin receptor signaling underlie neoadjuvant metformin administration in breast cancer: a prospective window of opportunity neoadjuvant study [114].
et al., 2104: Metformin-mode of action and clinical implications for diabetes and cancer [51].
Lega et al., 2014: The effect of metformin on mortality following cancer among patients with diabetes [54].
Thompson, 2014: Molecular pathways: preclinical models and clinical trials with metformin in breast cancer [24].

formin, unlike metformin, does not require the presence of the organic cation transporter 1 (OCT-1/SLC22A1), which is highly expressed only in hepatocytes [51], has a more ubiquitous uptake in tissues, and may be useful as a drug for activating AMPK directly in cancer cells [52]. Second, phenformin is a 50-fold more potent inhibitor of mitochondrial complex 1 than metformin and further increases the AMP/ATP ratio that promotes AMPK activation and signaling [48, 53]. In addition, some population-based studies have suggested that metformin reduces cancer risk when it is used in patients with diabetes compared to a control group [2, 3]. However, a recent meta-analysis found no significant benefit from metformin on mortality from breast cancer in patients with diabetes [54].

Other AMPK activators

An AMP mimetic, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), acts exclusively by facilitating an increase in AMPKα subunit phosphorylation at Thr172 by LKB1. The thiazolidinedione family of PPARγ agonists (rosiglitazone and troglitazone) stimulates AMPK by increasing the ratio of AMP/ATP in muscle cells [55], although this may not apply to MCF-10A non-malignant breast cells. Metformin and AICAR, but not thiazolidinediones, suppress estradiol-induced cellular growth [56, 57]. Resveratrol, at least in the brain, activates AMPK via CaMKK [58]. Honokiol is a polyphenol extracted from seed cones of magnolia species [59] that has been shown to increase cytoplasmic accumulation of LKB1 and AMPK activity, thereby inhibiting cell proliferation [60]. The tea polyphenols also promote activation of AMPK. Treatment of MDA-MB-231 breast cancer cell lines with the green tea polyphenol epigallocatechin gallate (EGCG) analogs 4 and 6 activate the AMPK pathway, resulting in a decrease in mammosphere formation, cancer stem cell population, and breast cancer proliferation in a dose-dependent fashion [61]. Recently, vitamin D has been shown to promote the anti-tumor effects of metformin in ERα$^-$ MDA-MB-231 breast cancer cells, possibly by activating the AMPK/mTOR-related pathway [62]. Proteasome inhibitors such as bortezomib have also been shown to activate AMPK [63]. The mechanism is indirect, via an increase in the AMPK activator CaMKKβ. The active metabolite of aspirin (salicylate) was recently identified as an activator of AMPK [64]. A-769662, a direct activator of AMPK, binds to the same site on the AMPKβ1 subunit as salicylate. Although A-769662 mimics the actions of AMP, it has no effect on binding of AMP to the Bateman domains of the AMPKγ subunit. Many other pharmacologic agonists of AMPK have been investigated [65] and have been described elsewhere [40].

AMPK inhibitors

Effective pharmacologic antagonists of AMPK include compound C (dorsomorphin), a small molecule, cell-permeable pyrazolopyrimidine derivative that acts as a reversible ATP competitive inhibitor of AMPK [66]. Adenine 9β-arabinofuranoside acts in a similar manner to inhibit AMPK activation [40].

Differential expression of AMPK subunits in normal/benign breast tissue

Both humans and mice contain two AMPKα isoforms (α1 and α2) [67]. AMPKα1 is present at much higher levels than AMPKα2 in human breast tissue. We observed that AMPKα1 mRNA levels are 10-fold higher than those of AMPKα2 in mouse mammary tissue (unpublished data). Both isoforms are also expressed in hyperplastic breast tissues and human fibroadenoma [68]. Thus far, no publications have reported the expression of the several isoforms of the β and γ subunits of AMPK in normal or benign breast tissues (**Table 1**).

Mammary tissue is composed of the epithelial, myoepithelial, and stromal (connective/adipose tissue) cells [69]. The epithelial cells of ducts and alveoli are embedded in the stroma, the main components of which are adipocytes and fibroblasts (preadipocytes) [5, 70, 71]. We found that phosphorylated AMPKα subunit is present in both mammary epithelial and stromal cells in mice (unpublished data). Phosphorylated AMPK (pAMPK) has also been observed in primary human breast epithelial cells and human preadipocytes from fresh breast tissues [72]. One study showed that AMPKα1, but not AMPKα2, is present in the cytoplasm of epithelial cells of human breast tissue by immunohistochemistry (IHC) [73]. In contrast, another study showed that normal human breast tissue contained a strong, uniform expression of

both AMPKα1 and AMPKα2 at the same levels within the ductal epithelia [68]. The expression patterns of different isoforms of the AMPKβ- and γ-subunits have not been established in epithelial cells or preadipocytes. AMPK expression and localization in myoepithelial cells also has not been determined. Since AMPK is expressed in the cytoplasm and the majority of components in the cytoplasm is lost in mature adipocytes during tissue processing for IHC, it is unclear whether α, β and γ subunits of AMPK are expressed in mature adipocytes of the breast adipose tissue based on IHC.

The expression of AMPK subunits in breast tumor tissue

AMPKα and pAMPKα (Thr172) expression was assessed in human breast cancers by Zhang et al. [74]. Both were present with a mixture of diffuse and granular cytoplasmic staining. Heterogeneous staining was shown between, as well as within, certain tumor cores for both markers, varying from weak to intense staining. High AMPKα expression was associated with improved local recurrence-free ($P = 0.019$), relapse-free ($P = 0.016$), and breast cancer-specific survival ($P < 0.001$). The phosphorylated form of AMPKα was not associated with survival outcomes [74]. However, other studies have shown that pAMPK was decreased by about 90% in tumor tissue of two cohorts of 354 primary breast cancer patients compared to normal breast epithelial cells. Phosphorylation of acetyl-CoA carboxylase, a direct target of AMPK, was significantly positively associated with pAMPK [68, 75]. In both cohorts, reduced pAMPK was also significantly associated with higher histological grade and axillary node metastasis. These findings show that AMPK is dysfunctional in primary breast cancer [68, 75]. Reduced AMPK signaling and the inverse relationship with histological grade and axillary node metastasis suggest that AMPK reactivation could have preventive and therapeutic potential in breast cancer. The lack of association of pAMPK with breast cancer in the Zhang et al. [74] study is inconsistent with other studies.

The expression levels of AMPKα1 and AMPKα2 in breast cancer tissue are different from those in benign tissues. In one study, AMPKα1 protein was expressed at the same levels in patient-matched adjacent normal epithelium (ADJ) and tumor tissue; however, AMPKα2 protein immunostaining by IHC was significantly lower, by 27%, in tumor samples compared to patient-matched ADJ samples, and by 37% compared to normal mammary epithelium [75]. Furthermore, AMPKα2 but not AMPKα1 expression was found to be significantly suppressed in all cancer grades compared to normal, hyperplastic, or fibroadenoma tissues [68]. Together, these data reveal a significant and widespread reduction of AMPKα2 expression in mammary epithelial carcinoma (**Table 1**). Exogenous expression of AMPKα2 subunit in MCF-7 xenografts restored their growth control mechanisms, reduced proliferation, and increased apoptosis. Clearly, AMPKα2 activity plays a crucial role in modulating cell growth signals and tumor development (45).

Mechanism of AMPK activity in the proliferation, apoptosis, and migration of breast cancer cell lines

Anti-proliferative effects of AMPK activation

Activated AMPK phosphorylates TSC1/TSC2 and Raptor, causing the inhibition of mTOR; as a consequence, the translation of many macromolecules and lipids required for cell growth is inhibited [42], including p70S6 kinase for protein synthesis and enzymes for the synthesis of fatty acids, triglycerides, cholesterol, and glycogen [4]. Inhibition of tumor suppressor LKB1 reduces AMPK phosphorylation and lifts the inhibition of TSC2 on mTOR activity, thereby upregulating its downstream kinases and enzymes; this leads to increased protein and lipid synthesis and increased cellular growth and proliferation without cell cycle regulation [76, 77]. AMPK activation can also inhibit the cell cycle by downregulating TSC2-mTOR and upregulating the p53-$p21^{WAF1}$ axis via phosphorylation of p53 and the cyclin-dependent kinase inhibitor $p21^{WAF1}$ [78, 79]. Moreover, AMPK tumor suppressive activity is increased by suppressing HIF1α and its downstream glycolytic genes, reversing the Warburg effect [80]. pAMPK may also act to suppress tumor growth and metastasis by decreasing expression of FOXO3a and E-cadherin via inhibition of Akt signaling [81], and by inducing GSK3β- and sirtuin-1-mediated downregulation of the oncogenic protein metadherin [82]. This is accomplished by overcoming c-MYC upregulation of

metadherin. In addition, under mild glucose starvation, AMPK has been shown to suppress rRNA transcription and cell proliferation by upregulation of the histone demethylase KDM2A [83]. Cancer metabolism itself may suppress the activity of AMPK because Akt, which is frequently overexpressed in cancer, is capable of phosphorylating AMPK at Ser485, reducing its activity. Thus, activation of PI3K or inhibition of PTEN will suppress AMPK by increasing Akt. This results in increased anabolic activity of tumor cells.

A possible protective effect of AMPK activation via metformin is related to the inhibition of aromatase activity, which decreases the conversion of androstenedione and testosterone to estradiol. However, the conversion of estrone and estrone sulfate to estradiol via 17β-hydroxysteroid dehydrogenase/steroid sulfatase is not affected by metformin [84]. In addition, overexpression of CYP1A1, which is common in breast cancer cells, may promote cancer proliferation in part through suppression of pAMPK [85]. Other cancer-specific mechanisms may also be involved in reducing the availability of AMPK [1].

AMPK activation by metformin exhibits its effects on cell proliferation in a dosage- and cell line-dependent manner [86]. MCF-7 and T47D ($ER\alpha^+/PR^+$), BT474 (ER^+, PR^+, and $HER2^+$), and BT20 and MDA-MB-453 (ER^-, PR^-, and $HER2^-$) breast cancer cell lines showed a decrease in cell proliferation in response to metformin treatment. Metformin caused a decrease in cyclin D1, which led to release of sequestered $p27^{Kip1}$ and $p21^{Cip1}$ and cell cycle arrest. On the other hand, the very aggressive triple-negative breast cancer (TNBC) cell line MDA-MB-231 (ER^-, PR^-, and $HER2^-$) was resistant to metformin's effect due to low levels of cyclin D1 [86]. Another study also showed that *in vitro* metformin treatment reduced the growth of $ER\alpha^+/PR^+$ MCF-7 cells by 82% and T47D cells by 96% when compared with untreated controls. However, the growth of TNBC cells (MDA-MB-435 and MDA-MB-231) showed only partial repression, by 40% and 29%, respectively. Clearly, TNBC cells are not as sensitive as $ER\alpha^+/PR^+$ cells to metformin treatment with respect to limiting cell proliferation [87].

Biguanides or other activators of AMPK may have an additional function in breast cancer cell lines that express elevated levels of HER2 (HCC1419 and SKBR3) or epidermal growth factor receptor (EGFR; HCC1806 and BT549) [88]. Jhaveri et al. showed that AICAR administration leads to decreased phosphorylation of HER2 and EGFR in such cells and it is 2- to 5-fold more effective in suppressing cell growth than in cells lacking HER2 or EGFR overexpression [89]. They identified amino acid sequences in HER2 and EGFR that are potential substrates for AMPK. The sonic hedgehog (Shh) pathway may also be involved in the effects of metformin on breast cancer growth [90]. Metformin inhibited recombinant human Shh-induced cell migration, invasion, and stemness, and reduced cell proliferation in a xenograft model. The effects were reversed by siRNA-mediated suppression of AMPK. Thus, metformin inhibits growth of breast cancer cells via several mechanisms, primarily the AMPK/mTOR signaling pathway. Anti-proliferative effects by biguanides in breast cancer cell lines occur via not only cell cycle arrest but also a pro-apoptotic effect [91, 92].

AMPK activation in breast cancer cell apoptosis

Activation of AMPK by phenformin or AICAR not only has dose- and cell type-dependent antiproliferative effects in breast cancer cell lines, but also has p53-dependent apoptotic effects [91, 93]. Activation of AMPK is essential for mitochondrial fragmentation [94], a process that promotes timely apoptosis. Mitochondrial fission factor (MFF) is a mitochondrial outer membrane receptor for DRP1, the cytoplasmic guanosine triphosphatase that catalyzes mitochondrial fission, and a substrate of AMPK [94]. In MCF-7, T47D, and MDA-MB-231 breast cancer cell lines, typical apoptotic morphological features (e.g., fragmentation into apoptotic bodies) were seen after AMPK activation, though these features were less pronounced in T47D cells compared to the other two lines. AMPK activation induced mitochondrial membrane depolarization, indicating apoptosis in all cell lines; phenformin had the strongest and AICAR had the weakest depolarization effect (phenformin > metformin > AICAR) [91]. It is apparent that the pro-apoptotic action of AMPK is partially p53-dependent since it is seen, even if to a lesser some degree, in p53 mutated MDA-MB-231 cell lines, and it appears that

growth inhibition of T47D cells is more related to cell cycle arrest than to apoptosis [91]. On the contrary, phenformin treatment of xenografts of ERα$^+$/PR$^+$ MCF-7 and TNBC MDA-MB-231 cells caused a decrease in the number of mitotic figures without changing the number of apoptotic cells, suggesting an AMPK-induced cell cycle arrest leading to inhibition of the development and growth of these breast cancer xenografts [95]. Cell proliferation marker Ki67 showed no significant decline in phenformin-treated xenograft models [95], indicating that the Ki67 and mitotic activity index were discrepant in these xenograft models [96].

AMPK activation in breast cancer cell migration

AMPK indirectly inhibits the activity of p70S6 kinase, resulting in inhibition of actin cytoskeleton reorganization and inhibition of cell migration. Treatment of MCF-7 and MDA-MB-231 xenografts with Honokiol led to a dose-dependent inhibition of migration and a significantly decreased rate of tumor growth, tumor size, and weight compared with the control group [60]. The growth of HCC1806 (EGFR$^+$) breast cancer cells with an LKB1 homozygous mutation was not inhibited by Honokiol, which demonstrates that LKB1 is necessary for Honokiol modulation of AMPK-mediated growth inhibition [60]. Honokiol treatment also inhibited FAK phosphorylation and associated cell motility in breast cancer cell lines [60].

Oncogenic activity of AMPK activation

Recently, the oncogenic activity of AMPK was studied in relation to metabolic stress (hypoxia, nutrient deprivation, and matrix detachment). Under conditions of nutrient deficiency, pAMPK can provide necessary ATP by increasing fatty acid oxidation. Fatty acid oxidation produces increased NADPH levels, and utilization of NADPH is decreased by AMPK phosphorylation of ACC. The resulting increased levels of NADPH reduce the formation of cytotoxic ROS and increase cancer cell survival [17]. Another important mechanism by which AMPK may promote cancer progression involves the activation of ULK1, the autophagy initiating kinase, under conditions of nutrient restriction [97]. Some level of autophagy allows the cells to persist in a dormant state until nutrients are available [98, 99]. Autophagy is regulated by opposing effects of AMPK and mTOR: active mTOR inhibits ULK1 in nutrient-replete conditions and active AMPK stimulates ULK1 in nutrient-deficient conditions. When ATP is deficient, pAMPK predominates and autophagy will be initiated, allowing the cancer cell to survive. If the expression of LKB1 or AMPK is very low in a cancer cell, energy levels may be insufficient for survival. In this case, AMPK activation and autophagy would help cancer cell survival. Therefore, AMPK inhibitors, rather than activators, may work well with drugs that increase metabolic stress to inhibit tumor growth [1]. Although salicylate is a direct activator of AMPK, in the absence of AMPK gene expression, salicylate has a negative effect on cell survival through increased CYP2E1 activity and ROS formation [100]. In MYC-driven cancers such as TNBC, MYC-dependent growth strains cellular energy resources and stimulates AMP-activated kinase to provide energy [101]. MYC promotes lipid, nucleotide, and protein synthesis; these biosynthetic processes deplete cellular resources and lead to activation of AMPK. This enhances the ability of the tumor cells to meet energy demands.

In summary, at early stages of tumor growth, when adequate energy is available, the beneficial effects of biguanides on tumor growth inhibition are likely dominant. After biguanide treatment, the balance of energy availability and protein and lipid biosynthesis is tilted in favor of energy availability and against biosynthetic processes needed for growth. Thus, a metabolic imbalance is created to suppress tumor growth. However, as the tumor grows and energy sources and nutrients become limited, biguanides may support tumor maintenance by increasing the availability of energy sources and promoting autophagy.

AMPK and human breast cancer

Breast cancer is the most common cancer among women in the United States (other than skin cancer). About 1 in 8 (12%) women in the US will develop invasive breast cancer during their lifetime. Breast cancer can be divided into four main molecular subtypes based on the presence or absence of biological markers: hormone (estrogen or progesterone) receptors (HR$^+$/HR$^-$) and excess levels of human epidermal growth factor receptor 2 (HER2$^+$/HER2$^-$), a

protein promoting breast epithelial cell growth [102, 103]. These four subtypes are luminal A ($HR^+/HER2^-$, 74%), luminal B ($HR^+/HER2^+$, 10%), HER2-enriched ($HR^-/HER2^+$, 4%), and triple-negative ($HR^-/HER2^-$, 12%) [104, 105]. The majority of breast cancers (84%) express estrogen and progesterone receptors, indicating the essential role of estrogen in breast cancer development. **Table 2** shows the effects of AMPK-activating biguanides on different types of breast cancer.

AMPK and aromatase in human ER^+ breast cancer

Estradiol, produced in breast tissue of postmenopausal women from androgen precursors [106], has proliferative and possibly genotoxic effects on breast epithelial cells [107, 108]. When the ovaries cease to produce estrogens in postmenopausal women, local aromatase expression in the breast preadipocytes (fibroblasts) and subcutaneous adipose tissue produce estrogens that drive ER^+ breast cancer development [109]. Both AMPK and aromatase are expressed in breast preadipocytes, indicating a potentially close relationship. Human aromatase gene expression is regulated by the transcription factors cAMP-responsive element (CRE)-binding protein (CREB) and/or CCAAT-enhancer-binding protein-β (C/EBPβ) [110, 111]. In human preadipocytes, AMPK activation decreases aromatase expression via decreased transcriptional activity of CREB [5]. AMPK activation also downregulates C/EBPβ expression in mouse preadipocytes [112] (**Figure 2**). Thus, the pAMPK pathway negatively regulates aromatase expression and estrogen formation in breast preadipocytes. Since aromatase inhibitors are widely used in postmenopausal women with breast cancer, there is a potential for synergism between an aromatase inhibitor and metformin; this possibility is under exploration in a Korean neoadjuvant trial [24]. In a preoperative trial of metformin in breast cancer patients, significant upregulation of pAMPK and downregulation of pAkt were demonstrated compared to controls [113, 114]. Tamoxifen is the primary adjuvant therapy for premenopausal women with ER^+ breast cancer, and the synergistic effects of metformin and tamoxifen have also been considered in a subgroup analysis of the ongoing MA32 adjuvant metformin trial [115] (**Table 3**).

AMPK in human $HER2^+$ breast cancers

There are four different members of the family of EGFRs (or ErbBs): ErbB1/EGFR, ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4 [116]. The HER2 isoforms are important factors that promote cell proliferation when energy supplies are adequate. Overexpression of the HER2 oncogene can be found in both luminal B and HER2-enriched breast cancers [117, 118].

ErbB2/HER2 in energy utilization: The activated ErbB2/HER2 receptor exerts proliferative roles via three major distinct pathways: Ras/Raf/MAPK, JAK/Stat, and PI3K/AKT/mTOR [117]. ErbB2/HER2 activation promotes synthesis of glycolytic intermediates used in synthesis of lipid, protein, and glycogen in order to maintain cell proliferation. Importantly, the PI3K/AKT/mTOR pathway is involved in energy regulation of cancer cells, and is opposed by the actions of AMPK. The AMPK pathway is not activated under normal conditions, when nutrient and energy supply is adequate for proliferating cancer cells. However, when energy is lacking, AMPK is activated by a high AMP/ATP ratio to promote energy (ATP) production. Meanwhile AMPK activation opposes the actions of PI3K/AKT/mTOR and inhibits the biosynthesis of macromolecules needed for cell proliferation. Thus, energy production is promoted at the expense of growth [117, 119, 120].

Combined anti-HER2 and biguanides treatment: Anti-HER2 treatment has been used over the last decade to enhance survival rates in both initial and metastatic stages of $HER2^+$ breast cancers [121]. The HER2 inhibitor trastuzumab is the currently approved treatment for the HER2 subtype of breast carcinoma; however, clinical practice shows that resistance develops within 1 year after start of the treatment [122]. HER2 inhibitor use is also associated with severe side effects of mitochondrial dysfunction, leading to cardiomyopathy in cancer patients [123, 124]. The underlying reason for this side effect is that ErbB2/HER2 is crucial for normal cardiac development and functioning [125, 126]. In order to overcome the serious cardiac side effects and lack of sustained efficacy of anti-HER2 treatment, the simultaneous inhibition of HER2 and activation of AMPK by biguanides has been suggested

based on the demonstrated cardioprotective effects of the biguanides [127].

Another HER2 inhibitor is lapatinib, which can block the ErbB2/HER2 pathway in treatment of HER2 breast cancer [127]. Lapatinib acts only in HER2-overexpressing breast cancer tissues, and not in cardiac tissue where HER2 is essential for appropriate functioning [127, 128]. As a result, the incidence of cardiomyopathy is lower with lapatinib compared to trastuzumab [3, 124, 127]. However, as with trastuzumab, resistance may also be a problem, as has been demonstrated at the molecular level by increased Akt and p70S6 kinase 1. Again, biguanides can be added to the lapatinib treatment regimen to downregulate the PI3K/Akt/mTOR pathway and prevent lapatinib resistance [117, 118, 129].

AMPK in human TNBCs

TNBCs are a very heterogeneous group of cancers and are usually associated with cytokeratin 5/6 and EGFR-positive staining. Since AMPK activation targets and suppresses Akt/mTOR, it has positive effects in all TNBCs. In addition, AMPK activation suppresses EGFR expression; phosphorylation of MAPK, Src, and STAT3; and expression of cyclin D1 and cyclin E [115, 130]. The ongoing MA32 adjuvant metformin trial includes patients with TNBC to test the effectiveness of biguanides in this group. A formal subset analyses of TNBC patients will further evaluate the anti-tumor effects of metformin in TNBC breast cancer cell lines.

Conclusions

AMPK activation decreases systemic glucose and insulin levels and at the same time directly inhibits mTOR, a central component in regulation of cell growth, reducing the proliferation of all breast cancer subtypes. AMPK-activating metformin and phenformin are expected to be novel therapeutics for breast cancer prevention and treatment via energy stress. However, the opposing actions of biguanides have yet to be resolved. In the review by Zadra et al. [1], AMPK is described as a conditional tumor suppressor or a contextual oncogene. Clinical studies cited therein reveal that the expression levels of pAMPK or acetyl CoA carboxylase 1 measured in tumors are generally associated with no difference or a favorable outcome, but in a few studies, higher levels of AMPK are associated with higher tumor grade or lower overall survival. Cells deficient in LKB1/AMPK are unable to restore ATP levels and are more susceptible to cell death. In the context of metabolic stress, AMPK inhibitors rather than activators may work well with compounds that cause metabolic stress. Observational trials of patients with diabetes generally show some positive benefit of metformin but case-control trials have not yet been concluded. To date, there is no evidence from clinical trials that treatment with metformin has a negative effect on cancer incidence in any setting. Survival outcomes with metformin therapy have been studied in patients with diabetes and breast cancer. In one study, Peeters et al. [131] found no difference in breast cancer-specific survival with metformin treatment, whereas another study He et al. [132] found a significantly lengthened survival (HR 0.52) among patients treated with metformin. More definitive results await the conclusion of case-control trials. Assuming positive results of these clinical trials, future studies will be needed to determine metformin dose and tissue availability, phenformin side effects, and interaction with the existing treatments for different types of breast cancers.

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Disclosure of conflict of interest

None.

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